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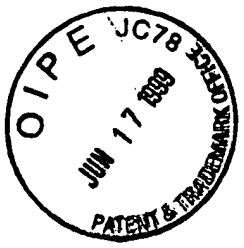
PATENT
Our Docket: SALK2190 6/22/99

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



In re Application of:
O'Gorman et al.

Serial No.: 08/919,501

Filed: August 28, 1997

For: SITE-SPECIFIC RECOMBINATION IN EUKARYOTES AND CONSTRUCTS USEFUL THEREFOR

Group Art Unit: 1633

Examiner: M. Wilson

CERTIFICATION UNDER 37 CFR §1.8

I hereby certify that the documents referred to as enclosed herein are being deposited with the United States Postal Service as first class mail on this date, 6/14, 1999 in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

Stephen E. Reiter, Reg. No. 31,192
(Name of person mailing paper)

Signature 6/14/99
Date

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF
APPLICANT UNDER 37 C.F.R. §131

Sir:

We, Stephen O'Gorman and Geoffrey Wahl, the joint inventors of the above-identified application, do hereby declare and state that:

We are familiar with the content of the above-identified application.

We are aware that claims 1, 2, and 4 of the above-identified application have been rejected for alleged anticipation under 35 U.S.C. §102 over Lewandoski *et al.* *(Current Biology* 7:148-151, 1997) (hereinafter "Lewandoski") and claims 1-2, 4-5, 10-16, 18-19 and 24-44 have been rejected for alleged obviousness under 35 U.S.C. §103(a) over a combination of references including Lewandoski.

Best Available Copy

The claimed invention was conceived and reduced to actual practice by us in the United States as joint inventors prior to the publication date of the Lewandoski article, as supported by the evidence which follows:

At the time the present invention was conceived and reduced to actual practice, Stephen O'Gorman was the leader of the research team that performed the experiments set forth as Examples 1 through 5 of the above patent application, which experiments were completed in laboratories of The Salk Institute for Biological Studies in La Jolla, California prior to the publication date of the Lewandoski article. In support of this statement are attached true copies (with only the dates redacted) of pages from laboratory notebooks belonging to The Salk Institute for Biological Studies, as follows:

1. A 652 bp fragment of the mP1 promoter was isolated by PCR using genomic DNA from CCE embryonic stem cells as a template (Lab notebook pages 1 through 5).

2. The mP1 promoter fragment was fused to a modified Cre coding sequence in the plasmid pOG304M (Lab notebook pages 6 through 10).

3. A Cre expression plasmid pOG231 was prepared by fusing a modified Cre coding sequence to a synthetic intron and CMV promoter. (Lab notebook pages 11 through 13).

4. A plasmid pOG277 containing a loxP-flanked neomycin cassette was prepared by inserting a wild-type loxP site into pBSKS (Stratagene) and then cloning a neomycin cassette between interations of this loxP site. (Lab notebook pages 14 through 16).

5. A hoxb-1 targeting construct containing a NruI site was prepared and the loxP-flanked neo cassette from pOG277 was inserted into the NruI site. (Lab notebook pages 17 through 29.

6. The P2Bc allele of Figure 1 was created by inserting a Lox-P flanked neomycin cassette and a β-GAL sequence into RNA polymerase II. (Lab notebook pages 45 through 61).

7. Transgenic mice were obtained from fertilized mouse oocytes injected with the protamine-Cre fusion gene from pOG304M (Lab notebook pages 62 through 65).

8. Heterozygous ProCre males were mated to wild-type females and the resulting progeny were examined by Southern blotting to determine the segregation pattern of ProCre nucleic acid constructs and P2Br alleles. (Lab notebook pages 30 through 44 and 62 through 67).

Thus, we maintain that the subject matter contained in the above-identified application was conceived and reduced to actual practice by us prior to the date of publication of the Lewandoski reference.

We further declare that all statements made herein of knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6/14/99
Date

Stephen O'Gorman
Stephen O'Gorman

6/14/99
Date

Geoffrey Wahl
Geoffrey Wahl

Attachments: Laboratory Notebook Pages 1-67.

OIPE JC78 JUN 17 1999 PATENT & TRADEMARK OFFICE

Protamine promoter PCR

FILENAME: PROTAMINE PROMOTER PRIMERS
IN: LAB/SEQUENCES/CRE/PROTAMINE

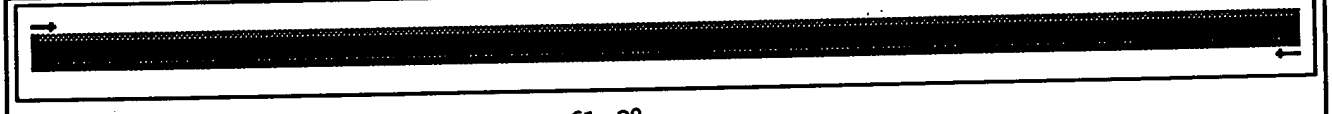
This is a pair of primers to amplify the mouse protamine 1 promoter from -560 to the translational start. The 5' primer includes a Bgl II site. The 3' primer contains a SmaI site. Both of these sites are meant to allow us to drop the PCR product into pOG234. The PCR product should be about 570 bp in length.

```
Primer positions: U 1    L 630
Upper/Lower: the most stable 3'-dimer: 2 bp, -1.3 kcal/mol
  5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3' -> GMC 310
                          : : : :     ::   ||
   3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCCATC 5'

Upper/Lower: the most stable 3'-dimer: 3 bp, -3.2 kcal/mol
  5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3'
                                 |||
                              3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCCA

Upper/Lower: the most stable dimer overall: 5 bp, -9.4 kcal/mol
  5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3'          \
                        : :     ::  |||||
                    3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCCATC 5'
```

```
Lower Primer, 38-mer [630]:
Lower Primer: the most stable 3'-dimer: 3 bp, -3.2 kcal/mol
  5' CTACCCGGGTGCTGGCTTGGCCGGGAGCTGGCTCAGAG 3' --> GMC 311
                                       ||| :::
                                    3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGG

Lower Primer: the most stable dimer overall: 8 bp, -18.6 kcal/mol
                                5' CTACCCGGGTGCTGGCTTGGCCGGGAGCTGGCTCAG
                                   ||||||||
  3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCCATC 5'

Hairpin: ΔG = -8.0 kcal/mol, Loop = 12 nt, Tm = 98°
            5' CTACCCGGGTGCTG-
                    ||||||    )
  3' GAGACTCGGTCGAGGGCCGGTTCG-
```

Protamine promoter PCR

```
Upper Primer, 37-mer [1]:
Upper Primer: the most stable 3'-dimer: 2 bp, -1.3 kcal/mol
  5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3'
                           || ||
                        3' TGACTCCCTCCACAACCTGTAATGATCTGTCTAGAAG 5'

Upper Primer: the most stable dimer overall: 6 bp, -7.9 kcal/mol
                              5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAG
                                    ||||||
  3' TGACTCCCTCCACAACCTGTAATGATCTGTCTAGAAG 5'

Hairpin: ΔG = 1.3 kcal/mol, Loop = 4 nt
                           5' GAAGATC
                                 |||
  3' TGACTCCCTCCACAACCTGTAATGATCTGT
```

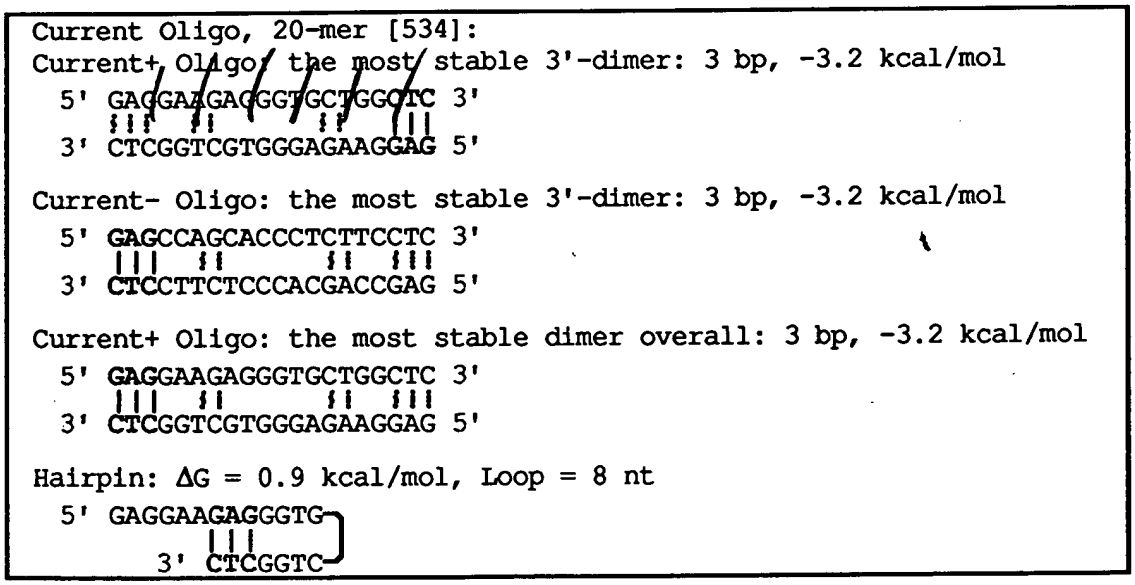


```
Optimal Annealing Temperature:    61.3°
Product length, GC content & Tm: 667 bp, 52.5% GC, 80.4°
Product Tm - Upper Primer Tm:     13.7°
Primers Tm difference:            17.5°
Upper Primer: 37-mer, pos. 1, Tm = 66.7°, 3'-pentamer ΔG = -6.4 kcal/mol
Lower Primer: 38-mer, pos. 630, Tm = 84.1°, 3'-pentamer ΔG = -6.7 kcal/mol
Salt & DNA Concentrations (fixed in this option): 50 mM and 250 pM, respectively
```

NOFILE

this is a seqeuncing primer for the protamine promoter. It is from the top strand of the promoter starting at 130 bp from the protamine translation initiation codon and extending for 20 bp.

It is oligo **319**



PCR REACTIONS TO MAKE SST PROTAMINE PROMOTER

Primers: 310
311

Resuspend in 200 λ TE
Read 260/280 on 1:100 dilution
Concentration = .2x A260

A320	A280	A260	280/260	260/280	
0.0047	0.5788	1.0482	0.5501	1.8177	310
0.0090	0.7395	1.3386	0.5494	1.8202	311

Primer mix

310: 10 δ ÷ 2.1 δ/λ = 4.8 λ
311: 10 δ ÷ 2.7 δ/λ = 3.7 λ
91.5 λ H_2O to Vf = 100 λ

Reactions

Want to use ~ 1δ CCE DNA/reaction, have stock that is 0.66 δ/λ
Want to run 8 reactions
Mix for 10 reactions

15 λ DNA
235 λ TE

Boil DNA 5 minutes
Place on ice immediately

THESE REACTIONS WERE USED IN THE CONSTRUCTION OF PLASMIDS 304 and 305

GOOD PRODUCT OBTAINED (SEE NEXT PAGE)

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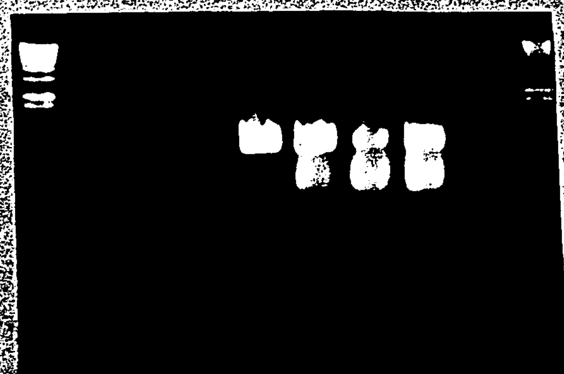
PCR Reactions to Clone Protamine Promoter

#	DNA	50mM $MgCl_2$	NTP	100ng/λ Primers	H_2O	2X PCR
1	25λ	2	2	1	20	50λ
2		2	2	3	18	
3		2	2	5	16	
4		1	1	3	20	
5		3	3	3	16	
6		4	4	3	14	
7		5	5	3	12	
8		3	3	0	19	

1. label tubes and place on ice
2. Add 2X PCR
   DNA
   Mg & NTP
   Primers
3. Add 0.5λ Taq polymerase
4. Mix by trituration.



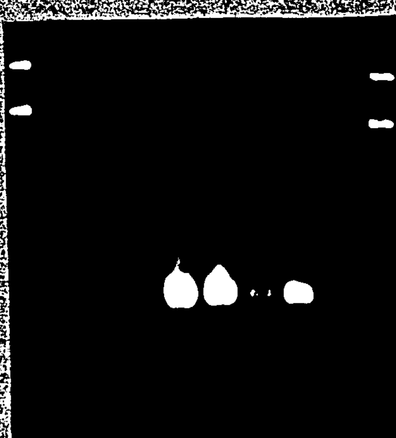
Cycling Parameters

1X 94°C, 60 sec

35X 92°C, 30 sec
55°C, 1 min
72°C, 72°C

5/12/95 10λ each reaction run on a standard minigel.

#4 = best



I am not pleased by the relative amounts of product in the various lanes. There is no good reason why #4-6 should show lots of product and lanes 1-3 should show none.
It is reasonable that #4 should be the cleanest reaction (1mM $MgCl_2$).

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① Clean up and ppt PCR reaction #4 product. ~5

- add 180 λ TE
- extract with 200 λ p/c
- add 20 λ 3M NaOAc
- add 1 λ glycogen
- add 300 λ EtOH
- 15 minutes ice
- 30 minutes TOMY
- wash pellet 70%
- resuspend in 20 λ

② Digest PCR product with BglII and PspAI PCR #4 only.

20 λ DNA
20 λ 10x #3
10 λ PspAI
10 λ BglII
140 λ H_2O

③ Digest pOG234 with BglII and SalI:

7.5 λ DNA = 3 δ (QP at 0.4 δ/λ)
20 λ 10x Sal
10 λ SalI
10 λ BglII
152.5 λ H_2O to VT = 200 λ

④ Digest pOG234 with PspAI and SalI

7.5 λ DNA = 3 δ
20 λ 10x Sal
10 λ SalI
10 λ PspAI
152.5 λ H_2O to 200 λ

⑤ Digest pOG231 with EcoRI + Klenow

1.7 λ DNA = 3 δ (prep at 1.8 δ/λ)
10 λ 10x Eco
5 λ EcoRI
83.3 λ H_2O to 100 λ

one hour — 30 min RT
+ 9.1 λ 2mM dNTP's — 65°C 5 min
+ 2 λ Klenow — bring to 200 λ

Extract with p/c ppt

(2)

(6) Digest PCR reaction #5 with SmaI (RT)

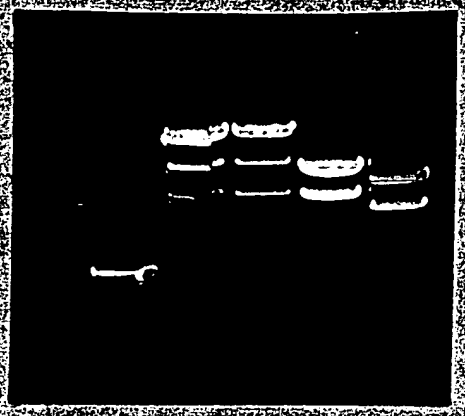
10 µl DNA
20 µl 10× #3
20 µl SmaI
150 µl H_2O

Extracted all 5 digests 1× with p/c, precipitated.

(7) [illegible]

(8) [illegible]

(9) [illegible]



LMA gel

① PCR with BglI and BglII

②+③ 234 with BglII + SalI

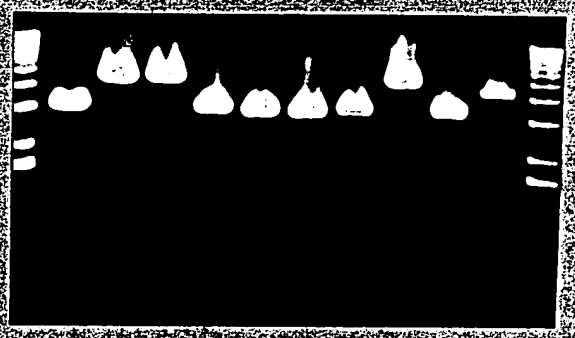
④+⑤ 234 with BglI + SalI

304 L1 : 7.5λ PCR gel
7.5λ 234 1.8Eb
5λ 234 vector

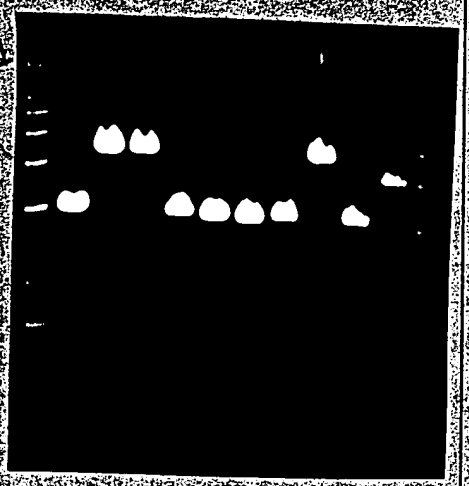
304 L2 5λ Blank
5λ 234 vector

Transformed with pG-1
Good transformation rate

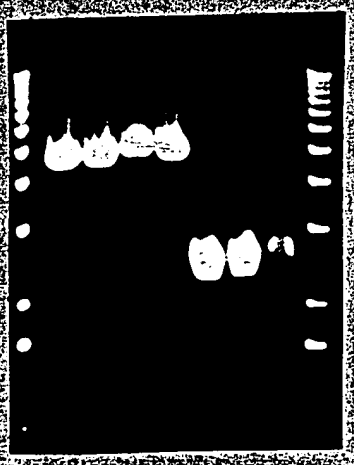
12 colonies digest with PstI #2, 3 gel looks good



RL#2, 3 saved



further checking digest: RL#2, 3



304 RL#2 & #3
SmaI
BglII
BglII + HindIII
234 (100ng) BglII + HindIII

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Digest pOS304 with SmaI and PstI (want ~3kb band)

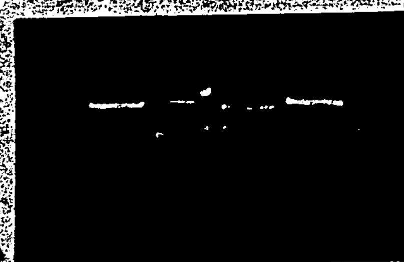
0.6 λ DNA (18) Cat 1.9 λ/λ)
5 λ 10x #4
4 λ SmaI
1 λ PstI
40 λ H_2O (to 50λ) 15,35 -> 0.6% LMA gel

Digest pOS306c with SmaI and PstI (want 1600bp band)

0.5 λ DNA (18)
5 λ 10x #4
4 λ SmaI
1 λ PstI
40 λ H_2O (to 50λ) 15,35λ -> 0.6% gel

Ligations

304 306



L1	5λ	304 gel	L2	=
	15λ	306 gel	15λ	blank
	12λ	10x gel		=
	1λ	ATP		=
	1λ	T4 ligase		=
	86λ	10mM Tris		=

14°C O/N

Ligations transformed
Slightly more in L2 than L1. 18 picked
Rapids digested with Sma & BamHI in #4, run on 1% gel

#11 used for QP.

2/9

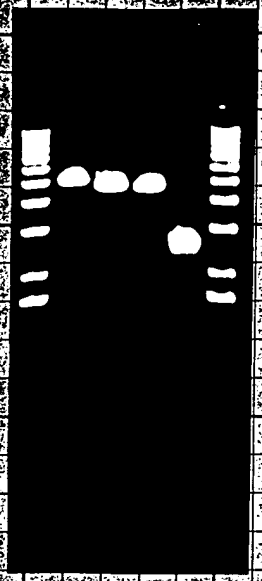
Do checking digests of 304 M

#3 (1) Bgl II

25°C. #4 (2) Sma I

Sal (3) Sal I

(4) Bgl II + Sal I (Sal buffer)



Qiagen Preps.

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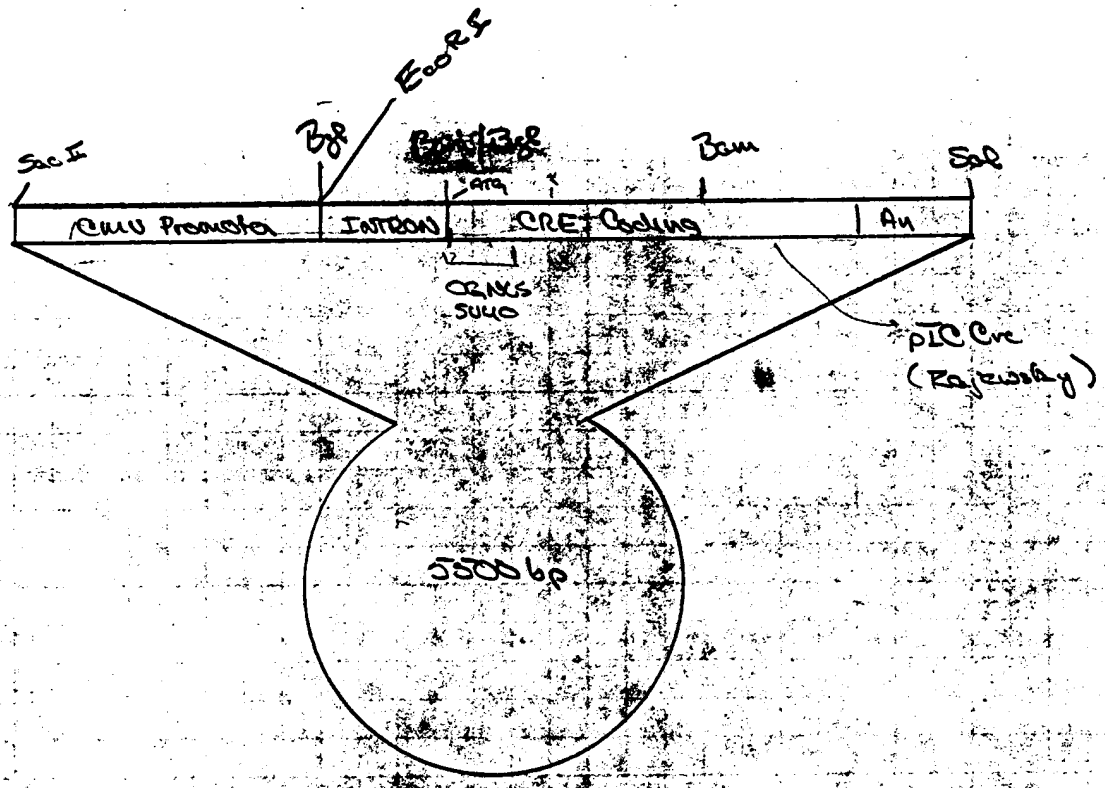


Vector = pCS4H ΔBB cut with BglII and SalI

Insert = pCS234 cut with BglII and SalI

① Digest 234 with BglII:

DNA	30λ
10x3	20λ
BglII	20λ
H_2O	130λ

All three prep digested one hour at 37°C. Then extracted with phenol/chloroform and precipitated.

② Digest 44ΔB with BglII

DNA	25λ (2.5δ)
10x3	10λ
BglII	7λ
H_2O	80λ

DNA spun down, washed

234 up in 30λ
44λB up in 25λ
ΔBB up in 30λ
} 1λ each onto mini

③ ~~Digest~~ 44ΔBB with ~~BglII~~

DNA	2λ (3.2δ)
10x3	10λ
BglII	7λ
H_2O	51λ

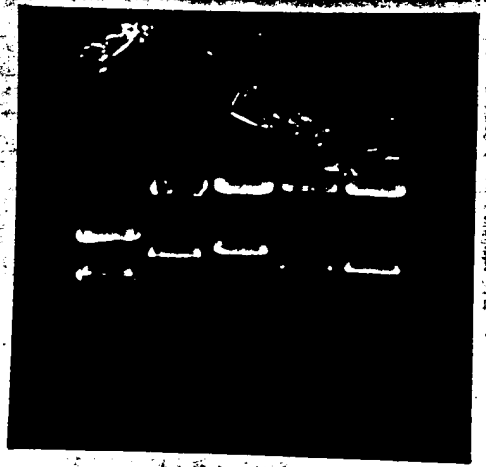
SHOULD HAVE CUT ΔBB with BHM

SalI digests

234	DNA	30λ
	H_2O	10λ
ΔB	DNA	25λ
	H_2O	15λ
ΔBB	DNA	30λ
	H_2O	10λ

all + 5λ Sal buffer
5λ SalI
one hour at 37°
2λ → mini gel
balance → LMA gel

From ΔB expect
3911 + 2011
From ΔBB expect
3911 +



LMA gel (0.6% LMA in TAE)

1) 234, lower band excised
2) 44ΔB, upper band excised.

Ligations

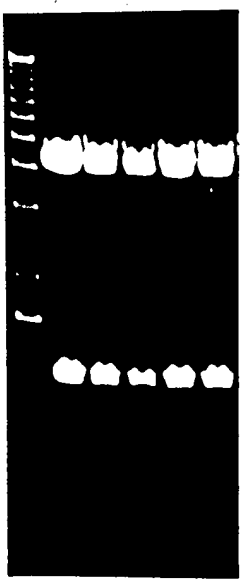
#1	15λ 234 gel	+ 86λ 10mM tris
	5λ 44ΔB gel	12λ 10x gel
		1λ ATP
		1λ ligase
#2	15λ blunt gel	
	5λ 44ΔB gel	to 14°C @ 10PM

3/12

Transformations: 5λ each mix into AG-1

Excellent transformation ratio obtained.

Colonies picked for rapid lysates.



Std rapid lysates, U+=50λ, 1λ for rapids
← Sac + Bam Digests. All give expected patterns

#1 & 2 saved
#1 selected for Qiagen

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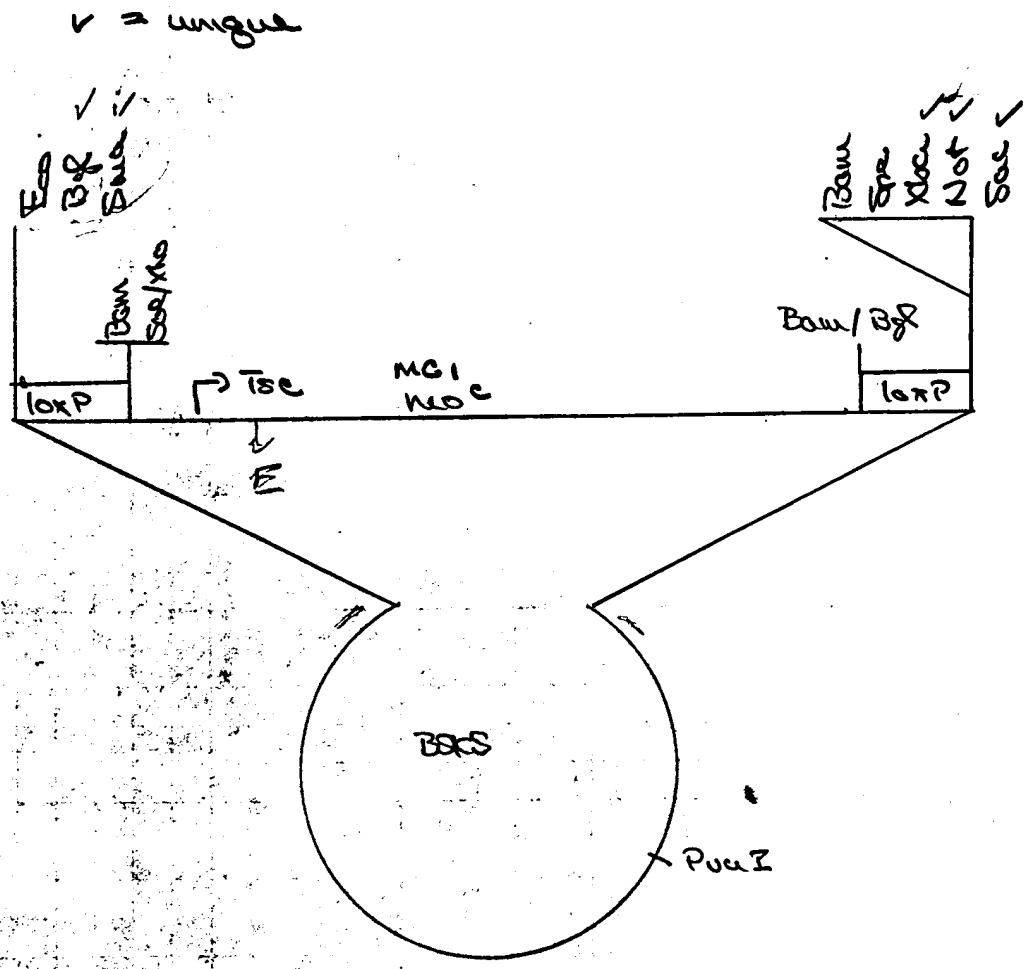


PvuI to BglII from pOG249B = 1200bp

PvuI to SalI from pOG249S = 1659bp

XhoI to BamHI from pMCIneoBglA = 11336bp

① 1° digest 249B (RL#1) and 249S (RL#1) with ScaI

20λ DNA
10λ ScaI
20λ 10x #3
150λ H_2O

5λ → minigel
p/c extract
EtOH ppt with glycogen and NaOAc O/N
Wash pellet
Resuspend in 20λ TE

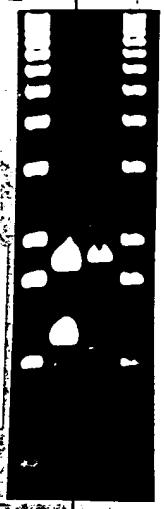
② 2° digest 249B with BglII

20λ DNA
5λ 10x #3
5λ BglII
20λ H_2O

③ 2° digest 249S with ScaI

20λ DNA
5λ 10x ScaI
5λ ScaI
20λ H_2O

5λ → minigel
15λ and 35λ → 0.6% TAE minigel. Run 2 hours



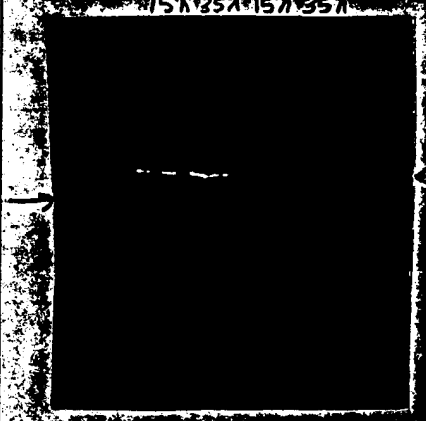
249B ScaI/BglII 5λ
249S ScaI/SalI 5λ

Excised bands.

249B: ~1200bp band (lower)
249S: ~1700bp band (upper)

LMA gel

249B		249S	
15λ	35λ	15λ	35λ



Ligations

277L1
5λ 249B gel
5λ 249S gel
10λ MetMo gel (7/11)
+86λ 10 mM Tris 7.4
12λ 10x gel ligation
1λ 100 mM ATP
1λ T4 ligase
— 14° O/N —

277L2
5λ 249B gel ( )
5λ 249S gel ( )
10λ blank gel

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5λ digestion used to transform 50λ AG1

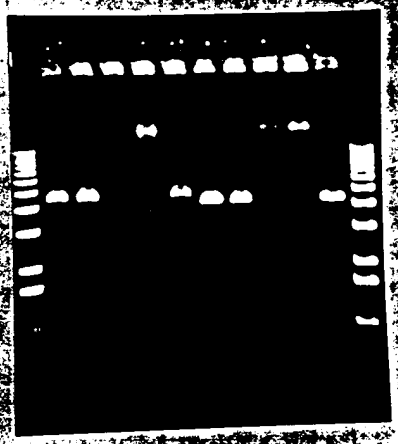
no colonies
5λ ligations → 100λ AG-1

Good colony ratio - low but OK #'s

plates left at 37° for 48 hr

12 "areas" picked into 5 ml LB + Amp.

Rapids: 5 cultures grown up, each digested with BamHI and with NotI



1-5 BamHI
1-5 NotI

#1 & 2 look good

#1 used for 293 transfection test for recombination

#1 used for Qiagen prep.

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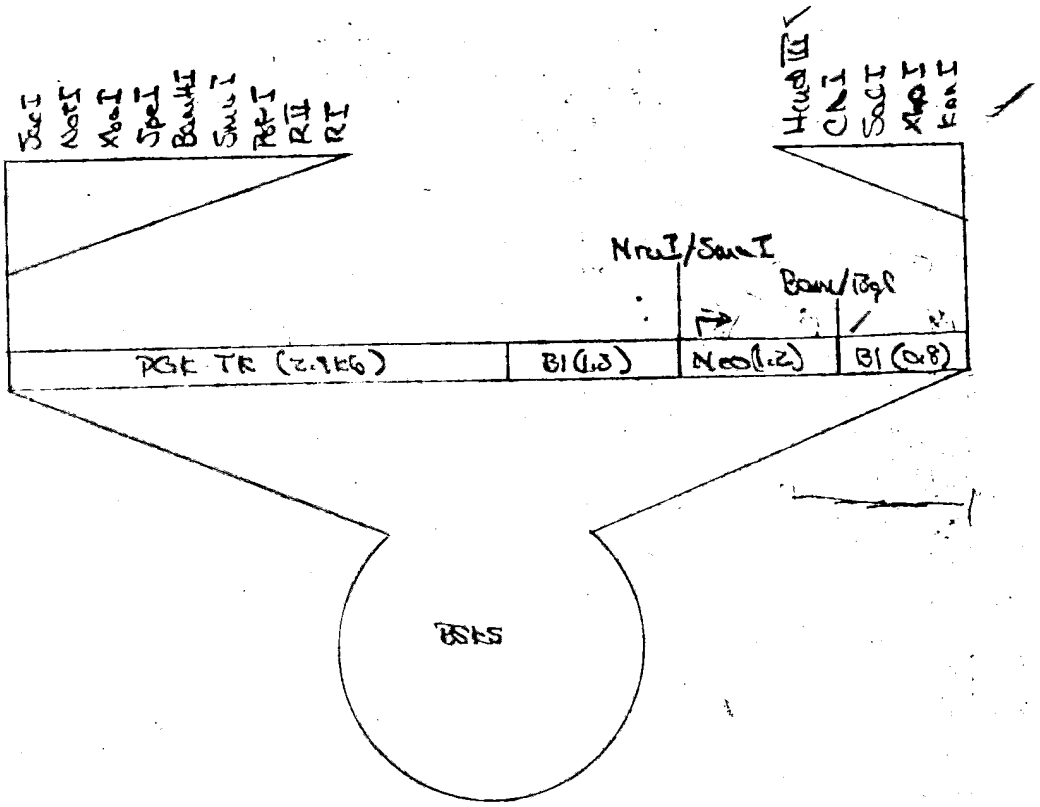
LoxP² N2O CASSETTE INTO PGK-TR + B1 Promoter



1) 336 NruI partial + HindIII
2) 336B BglII + HindIII
3) 277 BamHI partial + SacI

4.2	1.2	3.7	EcoRI
4.1	4.9		BamHI
1.0	2.4	5.5	SacI
			NotI
			SalI
			KpnI
			HindIII

① Digest 3δ pOG 336 with Nru I

16 λ 336 (12/5/95 prep at 1.9δ/λ)
20 λ 10X Nru I
5 λ Nru I
173.4 λ H_2O
200 λ

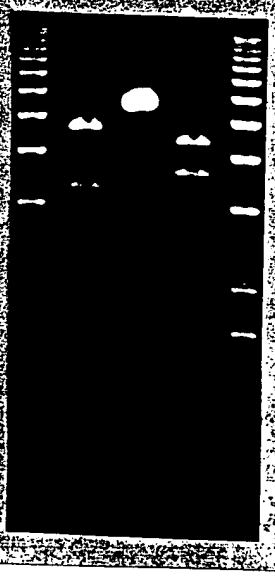
② Digest 10δ pOG313 with Nru I

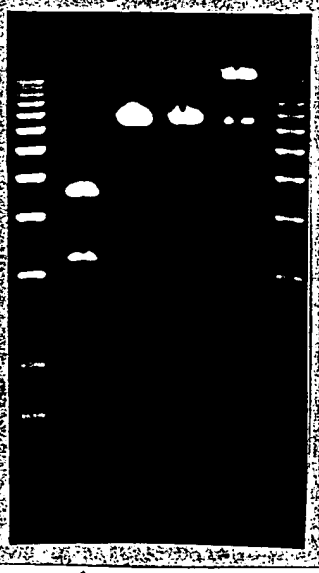
5.6 λ 313 (8/30 prep at 1.8δ/λ)
20 λ 10X Nru I
10 λ Nru I
164.4 λ H_2O
200 λ

③ Digest 5δ pOG 336 with Eco RI

2.6 λ 336
20 λ 10X Eco
10 λ Eco RI
167.4 λ H_2O
200 λ

One hour at 37°C.
Run 7 λ ①, 2 λ ②, 4 λ ③ on a minigel.
PLC, ppt all three overnight.





Nru I digest of 336 showed unmapped site in TK. Will need to do a partial

336	Nru I	7 λ
313	Nru I	2 λ
336	Eco RI	4 λ

336 (100ng)	Nru I	
	Not I	
	Kpn I	
342 (100ng)	Kpn I	

6/10

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① 10 µg 336 cut with Not I Vf = 200 µl / 10 µl enzyme

5.2 µl DNA (12.5 µg prep at 1.9 µg/µl)
20 µl 10x #3
10 µl Not I
164.8 µl H_2O

One hour at 37°C
2 µl → minigel
plc, ppt, spin 70% EtOH wash, resuspend in 20 µl TE.

② Cut EcoRI digested 336 with Not I
~~50% of~~ All resuspended DNA; 50 µl Vf, 5 µl enzyme
(my mistake)
20 µl DNA
5 µl 10x #3
5 µl Not I
30 µl H_2O

One hour at 37°C
1.5 µl → minigel

③ Partial EcoRI digest of Nru-cut 313
All of resuspended DNA
Vf = 200 µl
0.5 µl EcoRI
3, 6, 12, 24, 45 minutes
run 4 µl aliquots on docking gel

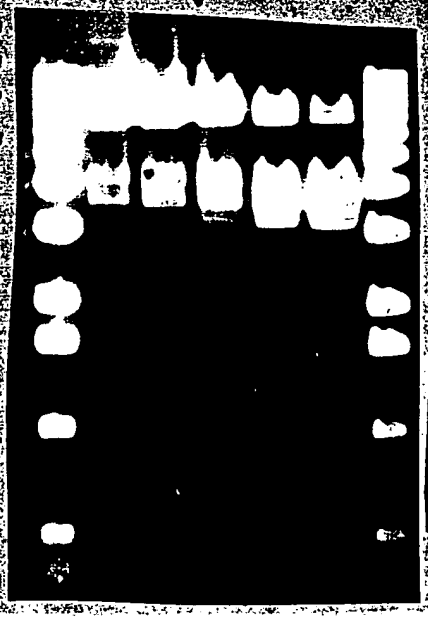
20 µl DNA
20 µl 10x Eco
5 µl Eco RI
159.5 µl H_2O

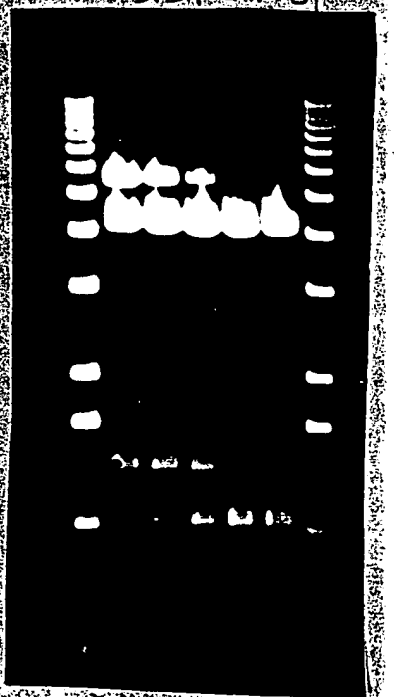
④ Partial Nru I digest 336 (Not I) with Nru I
All of resuspended DNA
Vf = 200 µl
0.5 µl Nru I
3, 6, 12, 24, 45 minutes
run 4 µl aliquots on minigel

20 µl DNA
20 µl 10x Nru
0.5 µl Nru I
159.5 µl H_2O

0.6% LMA / TAE gel

kb ladder
1. 313 partial 6 min
2. 30 µl 336 EcoRI/Not I
3. 336 partial 3 min
4. " 6 "
5. " 12 "
6. " 24 "
7. " 45 "





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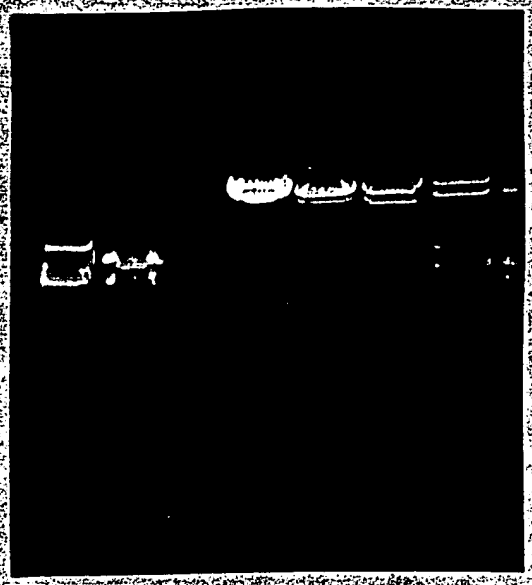


Min excised bands and set up ligations.

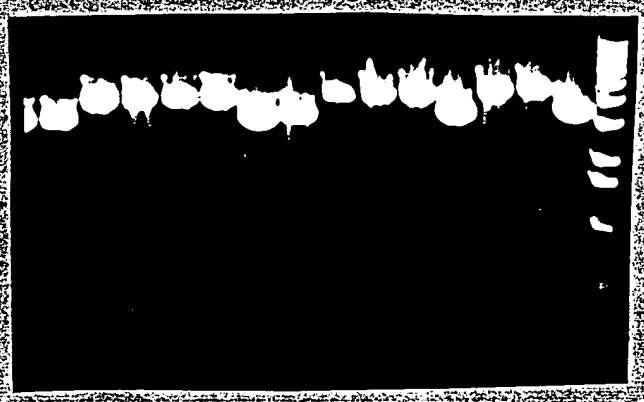
Ligations L1 and L2 were transformed into 100 λ of lab stock AG1's.

Transformation plates show about equal numbers of colonies on L1 and L2.

Picked 18 colonies for rapids tomorrow.

Standard Rapid lysates done - all 18 grew up.

Set up EcoRI digests



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National® Brand 42-182 100 SHEETS Made in U.S.A.

① NruI partial digestion of 258 pOG336

13λ DNA (12/5/95 prep at 1.9 γ/λ)
20λ 10X Nru
0.5λ NruI
166.5λ H_2O
200λ

To 37°C, timepoints taken at 3, 6, 12, 24, and 45 minutes.
(40λ → 4λ 250mM EDTA → ice)
1λ (125ng) of each timepoint ran out on a minigel.
P/C the rest of each timepoint and leave to ppt. overnight.

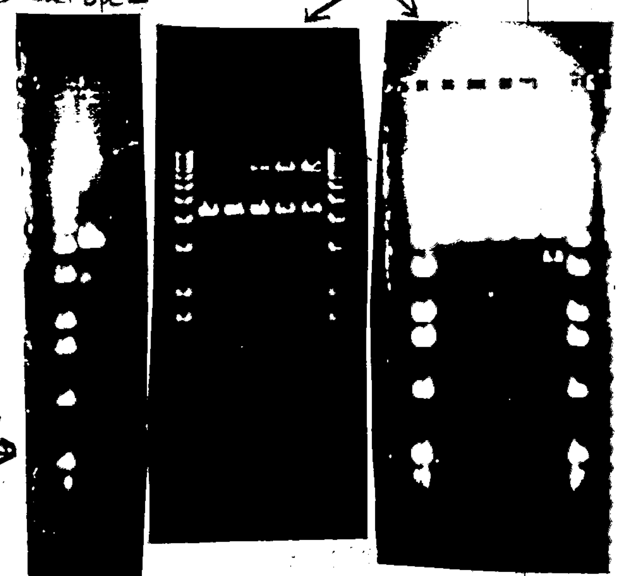
② SmaI + SpeI digest of pOG277

5λ DNA (7/30/94 prep at 1γ/λ)
20λ 10X #4
5λ SmaI
5λ SpeI
165λ H_2O
200λ

Added 5λ SmaI → RT for 30 min.
Added 5λ SpeI → 37°C for 30 min.
4λ → minigel →
P/C, ppt. overnight.

277 SmaI+SpeI

336 NruI partials



DNA was spun down for 30 min., washed with 70% EtOH, respun and resuspended in 20λ TE.

Redo SpeI digest of SmaI + SpeI digested 277

20λ DNA
20λ 10X #2
20λ SpeI
140λ H_2O
200λ

One hour at 37°C
4λ → minigel

\+ 8λ 2mM each dNTP
4λ Klenow

30 min. at RT
70°C for 5 min.

P/C, ppt.

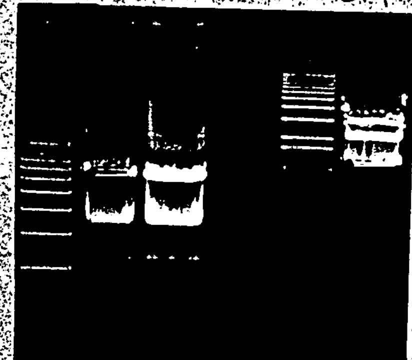
- CIP 336 NruI cut DNA 45' timepoint

20λ resuspended 336 DNA
5λ 10x #2
3λ CIP
22λ H2O

37°C 30', 15,35λ → 0.6% LMA gel

resuspend 277 SmaI + SpeI + Klenow DNA
in 20λ TE onto one lane of LMA gel

336 277



Ligations

L1 5λ 334 gel L2 =
15λ 277 gel 15λ blank

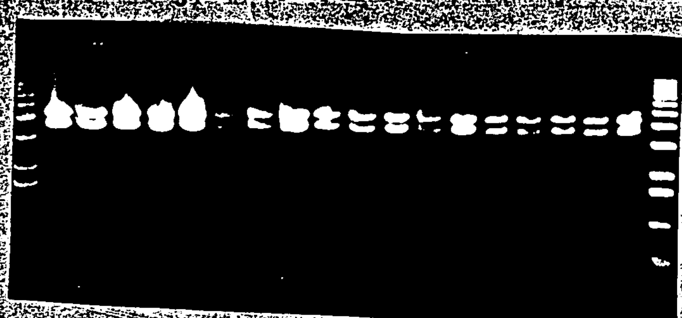
both 86λ 10mM Tris
12λ 10x gel buff
1λ ATP 100mM
1λ T4 ligase

14°C O/N

5λ → 50λ Ag-1, plated on LB+AMP plates

picked 18 colonies L1:L2 ratio good

RL#1-18 digest w/ EcoRI



- did not get wanted piece

- redo 343

① NruI partial digestion of 25δ pOG336

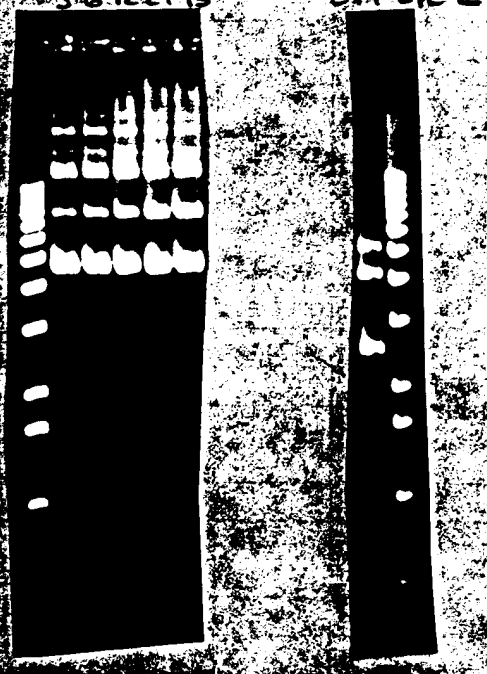
13λ DNA (12/5/95 prep at 1.9δ/λ)
20λ 10X NruI
0.5λ NruI
166.5λ H_2O
200λ

To 37°C, timepoints taken at 3, 6, 12, 24 and 45 minutes.
(40λ → 4λ 250mM EDTA → ice)
1λ (125ng) of each timepoint ran out on a minigel.
Plc the rest of each timepoint and leave to ppt. overnight.

② SpeI digest of pOG277

5λ DNA (7/30/94 prep at 1δ/λ)
20λ 10X #2
10λ SpeI
165λ H_2O
200λ

37°C for one hour
4λ → minigel
Plc, ppt. overnight



The partial turned out more incomplete than we would like - it would be better if there were less of the supercoiled and relaxed circular DNA and more of the doubly-digested DNA, but as long as we do not overload the gel and as long as we run the digest out sufficiently far, we should be O.K.

The SpeI digest of 277 was not very complete - there was a substantial amount of supercoiled and relaxed circular DNA. Steve spun down the DNA, washed it and redigested it with SpeI. He ran a small aliquot on a minigel and then cleaned up and precipitated the rest.

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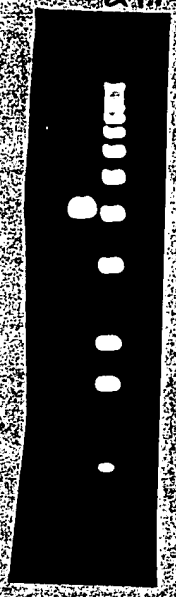
Redigestion of SpeI-cut 277 looks good

Spun down the SpeI-cut 277 and the 45 minute timepoint from the 336 NruI partial. Washed with 70% EtOH, respun, and resuspended both pellets in 20 λ TE.

Redigested SpeI-cut 277



CIP 336 NruI

20 λ DNA
5 λ 10x buffer
2 λ CIP enzyme
23 λ H_2O
50 λ

30 min. at 37°C
load 15 & 30 λ onto a 0.6% LMA/TAE gel
Ran for a long time
Want ~ 8 kb band

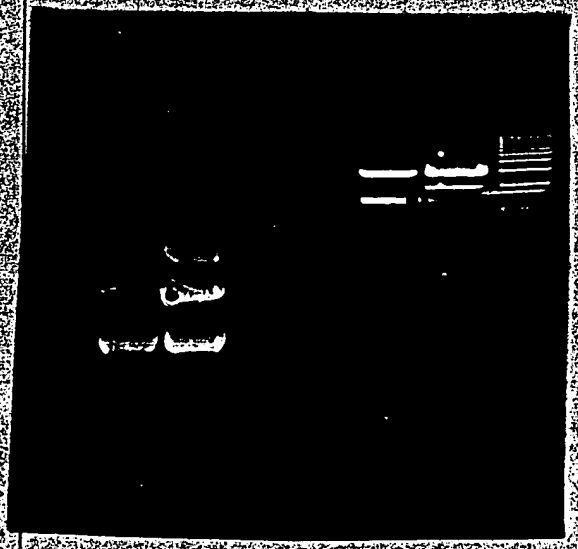
Klenow of 277 SpeI

20 λ DNA
2 λ 2mM dNTP's
5 λ 10x buffer
2 λ Klenow
21 λ H_2O
50 λ

30 min. at RT
70°C for 5 min.
PIC, ppt. spin, wash with 70% EtOH, respin, resuspend in 20 λ TE.

0.6% LMA/TAE gel

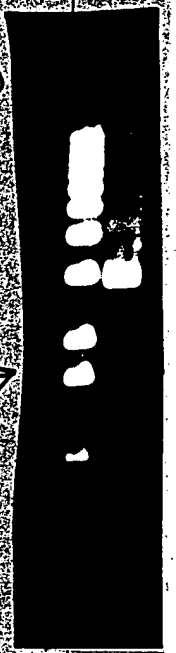
① kb ladder
② 336 NruI (CIP) 15 λ
③ " 30 λ
④ blank
⑤ blank
⑥ 277 SpeI (Klenow) SmaI 15 λ
⑦ " 35 λ
⑧ kb ladder



SmaI digest of 277 SpeI (Klenow)

20 λ DNA
5 λ 10x #4
5 λ SmaI
20 λ H_2O
50 λ

One hour at RT
1 λ → minigel
15 & 35 λ onto 0.6% LMA/TAE gel (Run ~ 1 hour)
Want ~ 1200 bp band



Ligations:

L1: 5 λ 336 gel, 15 λ 277 gel
L2: 5 λ 336 gel, 15 λ blank gel

BOTH:

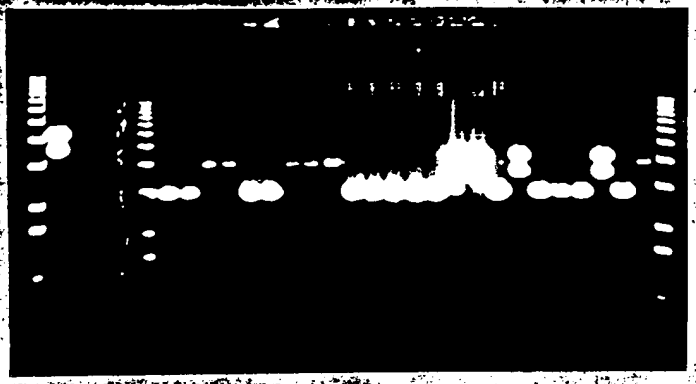
86 λ 10 mM Tris
12 λ 10x gel lig buffer
1 λ 100 mM ATP
1 λ T4 DNA ligase
To 14°C O/N

6/24

6/9/95 → Transformed 5 λ ligations into 50 λ Stratagene AG1's

- not many colonies on both L1, L2 plates
ratio good

Picked 24 colonies for Rapids



RL#1-24; 336. EcoRI
I do not believe that
any of these are correct.

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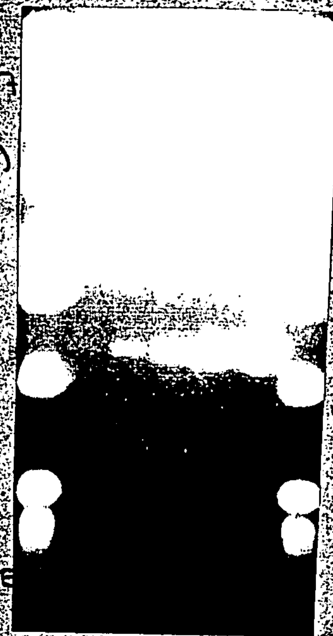
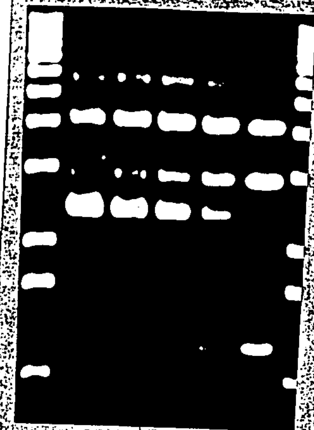
6/26

Construction

1. BamHI partial digest of 277

45λ DNA (0.8γ/λ 123095QP)
20λ 10x #3
1λ BamHI
134λ H2O
37°C
time point 3', 6', 12', 24', 45'
1λ of each → minigel
add 4λ 250mM EDTA
add 160λ TE
1x P/C, ppt w/ glycogen
spin 70% wash
resuspend 12' time point in 20λ TE





2. Sma complete of Bam partial 277

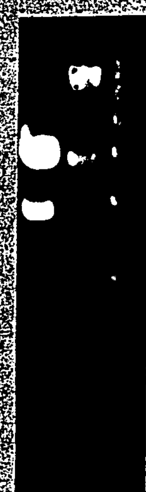
20λ DNA
4λ 10x #4
4λ Sma I
12λ H2O

RT 1hr, 1λ → minigel, 15, 30λ → 1% LMA gel

3. HindIII complete of NruI partial of 336
spin down 2
resuspend 1/2 96 NruI partial, 24' time point
in 20λ TE (see 336B notes)

20λ DNA
4λ 10x #2
4λ HindIII
12λ H2O

37°C 1hr, 1λ → minigel
15, 30λ → 0.6% LMA TAE gel



← 277 Sma + Bam(P)

336 HindIII + Nru(P)

4. BglII + HindIII digest of 336B

25λ 336B RL#1
5λ 10x #2
2.5λ BglII
2.5λ HindIII
1.5λ H2O
37°C 1hr, 1λ → minigel
15, 35λ → 1% LMA gel

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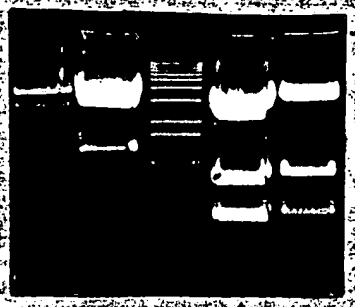
1% LMA gel



277 Bam(P) + Sma

336 Nru(P) + Hind →

336B Bgl II + Hind III

↑ 3 & 3B

ligations

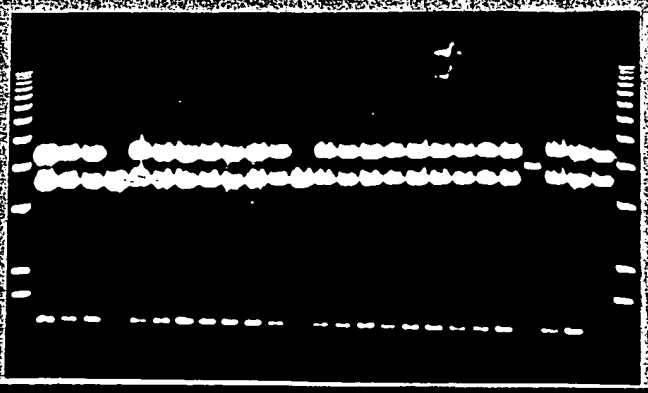
L1	3 λ	336 Nru(P) + Hind III gel	L2 :	=
	5 λ	277 Bam(P) + Sma I gel	10 λ	1.0% blank
	5 λ	336B Bgl II + Hind III gel		
both	12 λ	10x gel		
	1 λ	100 mM ATP		
	1 λ	T4 ligase		
	91 λ	10 mM Tris (7.4)		

14°C O/N

5 λ → 50 λ Ag-1 : plated w/ Amp

picked 24 colonies for rapid's ~ 6:1, ratio not good, a low number of colonies on both plates

343 RI #1-24 control 336 w/ Eco RI



RI #1-3, 5-11, 13-21, 23, 24 looks correct.

6/28

343 RL #1

BamHI

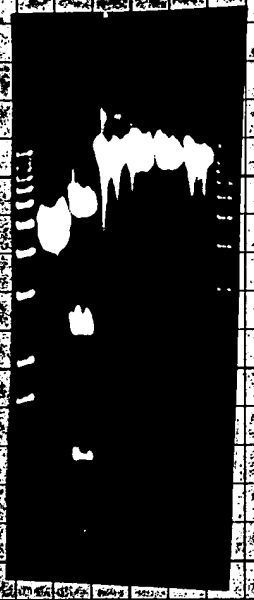
SacI

NotI

SalI

KpnI

HindIII



HOE ASSAY OF P2-S /304M | MOUSE TAIL DNA's

Standards.

Conc.	Read #1	Read #2
0	0	12
100	104	109
200	193	201
300	312	302
500	482	480
750	746	729
1000	1000	968

Samples. 3λ sample into 3ml assay buffer.

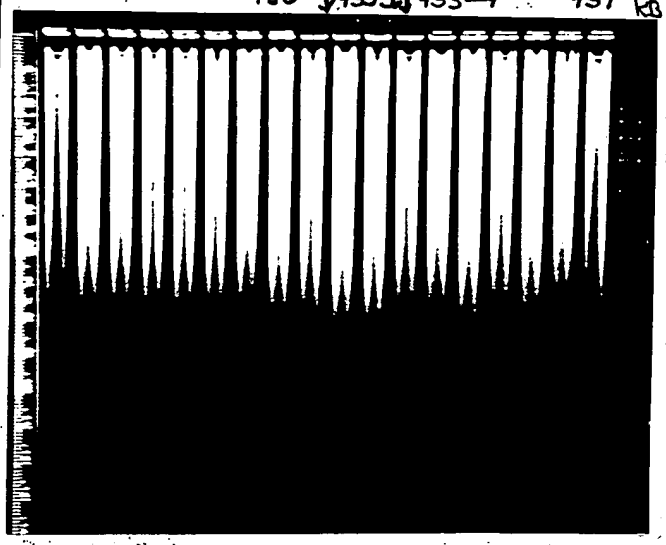
#	Conc. (ng/λ)	λ/10μg	10x(6λ)	Enzyme (6λ)	H_2O
722	342	29 λ	BamHI	BamHI	19 λ
723	360	28	↓	↓	20 λ
724	444	23			25
725	397	25			23
726	431	23			25
727	295	34			14
728	450	22			26
729	378	27			21
730	440	23			25
735	791	15	#3	PstI	33
736	694	15	↓	↓	33
753	294	34	#3	PstI	14
754	540	19	↓	↓	28
755	415	24			24
756	520	19			29
757	317	32			16
758	547	18			30
759	434	23			25
722		29	#3	PstI	19
723		28	↓	↓	20
724		23			25
725		25			23
726		23			25
727		34			14
728		22			26
729		27			21
730		23			25

1:40 pm

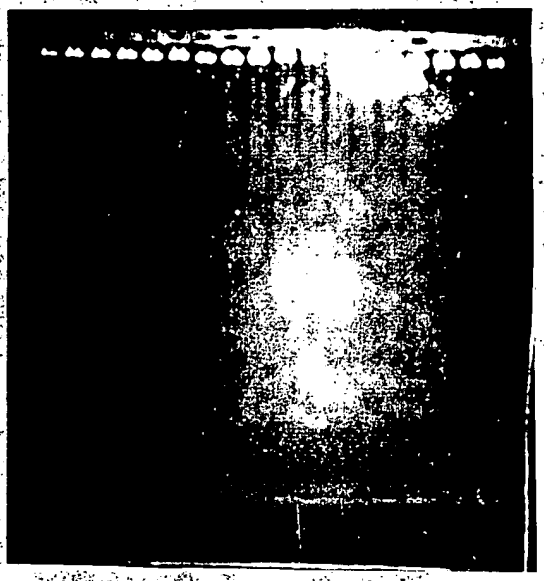
1, 7, 9, 10, 11

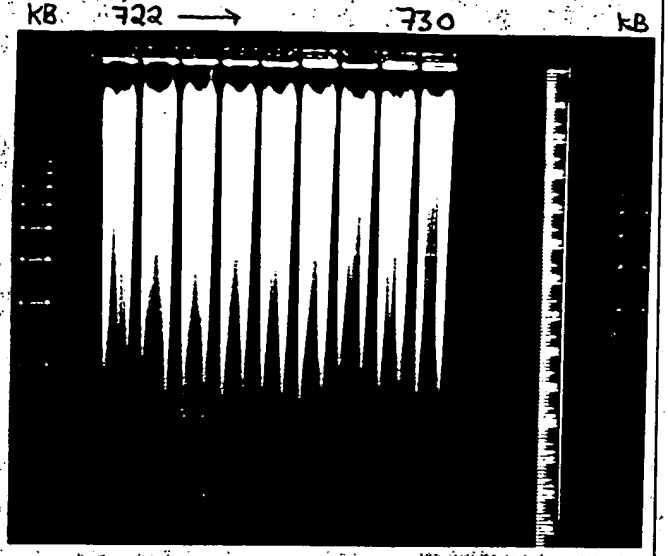
98/30

SOUTHERN HYBRIDIZATION OF P2-5/304 M MOOSE TAIL DNA's



Pst I digests 121695A
P2-5 P2-1 probe 12-18-95





BamHI digests 121695B
304M Cre probe

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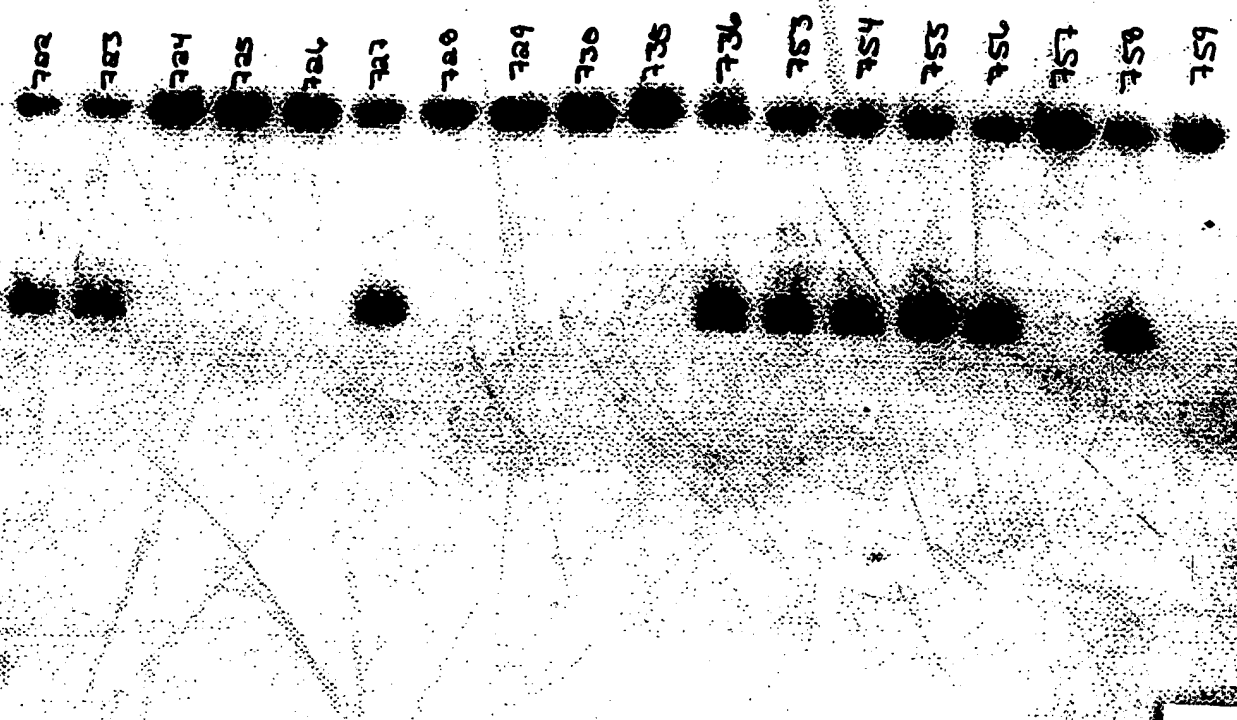
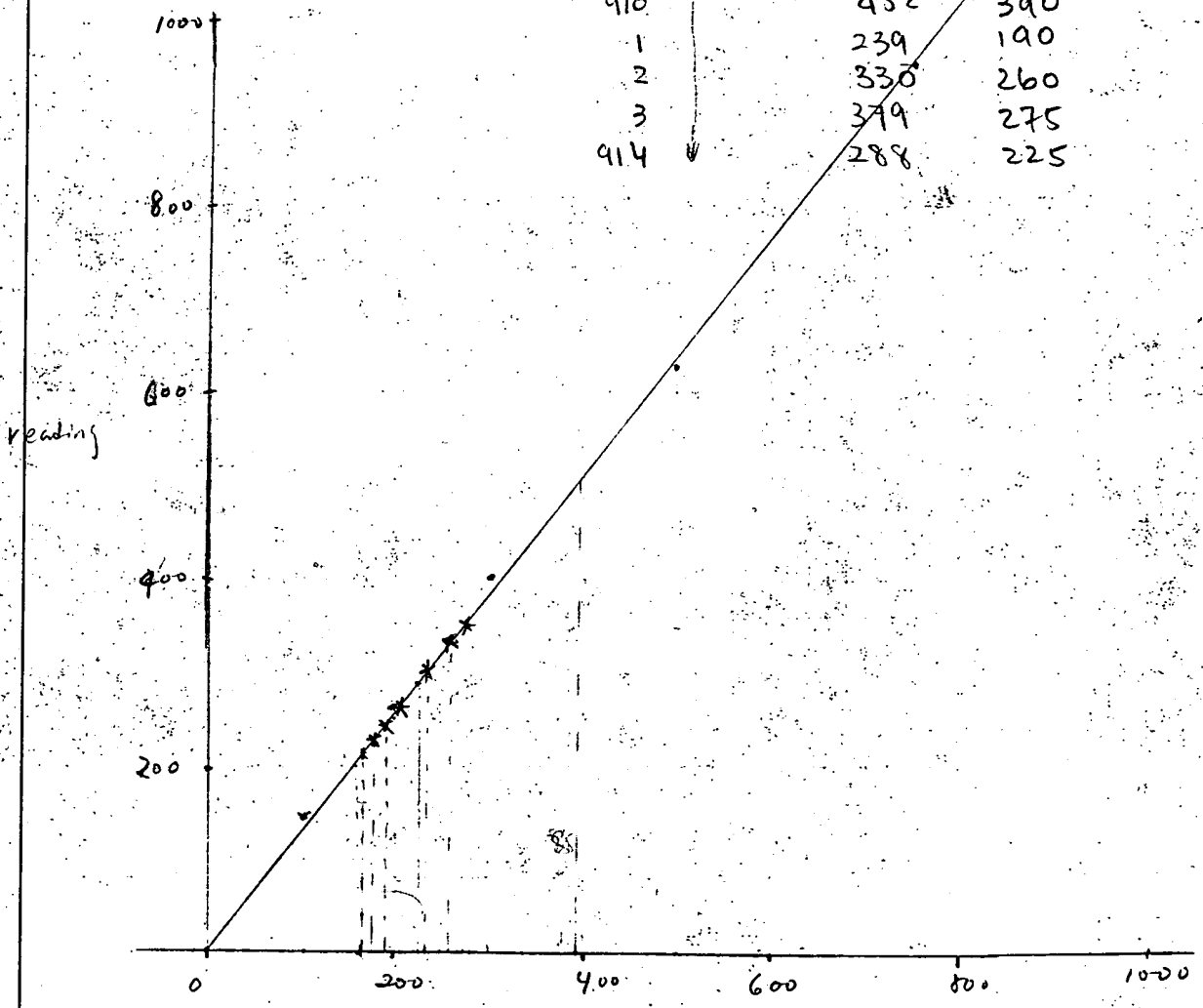


121695A
P2-5
P2-1 probe
24 hrs. exposure

Mouse DNA Quitification

Standard	reading
0	0
100	145
200	267
300	401
500	632
750	957
1000	1017

Sample #	reading	acc. ng/λ	
894*	346	280	
895	225	175	
6	264	200	mouse #894* - 914
7 (6λ)	329	260	Embryo.
8	242	190	
9	298	230	
900	261	200	
1	243	190	
2	248	190	
903 (6λ)	211	160.	
4 (9λ)	287	225	
5	298	230	
6	253	195	
7	295	230	
8 (18λ)	32	/	
9 (9λ)	203	180	
910	452	390	
1	239	190	
2	330	260	
3	349	275	
914	288	225	



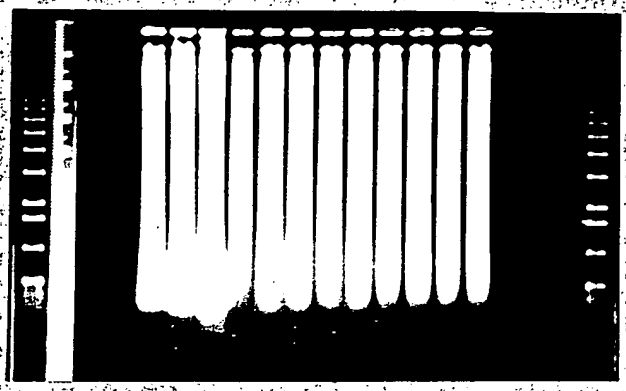
• - standards.
* - data

mouse Embryo DNA's genotyping digest w/ PstI

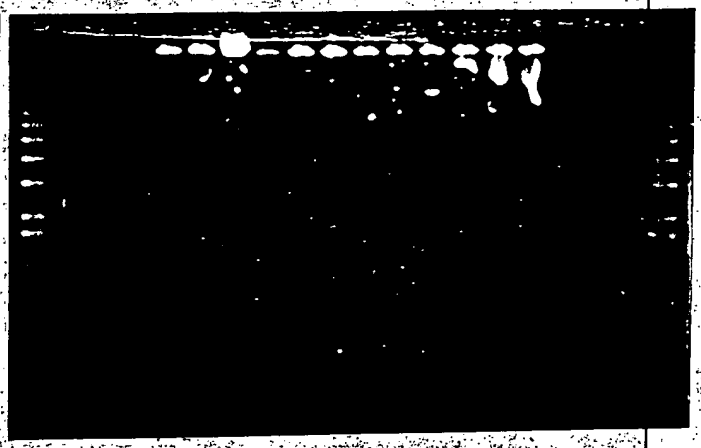
lane #		DNA conc. ng/λ	DNA λ	H_2O λ	10x #3 λ	Erg λ
1	1kb					
2-4	–					
5	TL	500	20	38	7λ	5λ
6	121495	600	17	41		
7	102595 #5	212	47	17		
8	894*	280	36	22		
9	895	175	57	3		
10	896 ✓	200	50	8		
11	897 ✓	260	38	20		
12	898 ✓	190	54	4		
13	899	230	44	14		
14	900	200	50	8		
15	901	190	54	14		
16	902 ✓	190	54	4		
17-19	–					
20	1kb					

22 v O/N

standard transfer, to prehyb for 8 hrs



P2-1 probe reading ~ 3x10⁵
used all ~100λ



gel c

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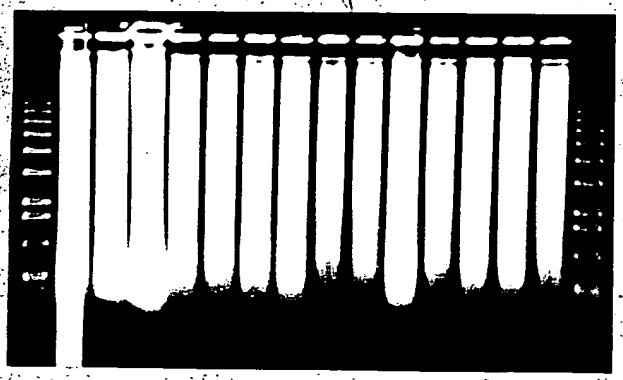
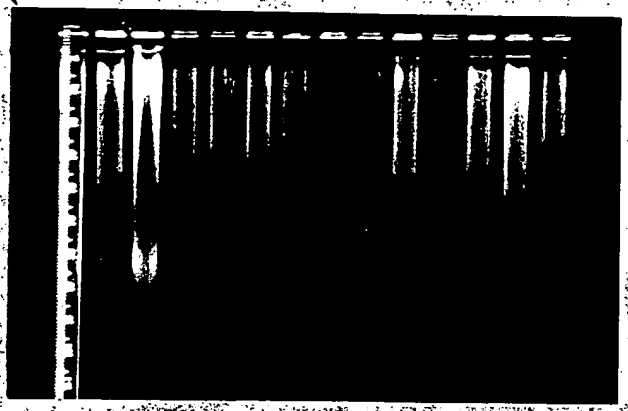
mouse Embryo DNA genotyping. digest w/ Pst I

lane#		DNA conc ng/λ	DNA λ	H_2O	10x #3	Enz.
1	1Kb					
2	TL	500	20	74	11λ	5λ
3	121495	600	17	77		
4	102595 #5	212	47	47		
5	903		90	ø		
6	904		↓	↓	↓	↓
7	905					
8	906					
9	907					
10	909					
11	910					
12	911					
13	912					
14	913					
15	914					
16	1Kb					

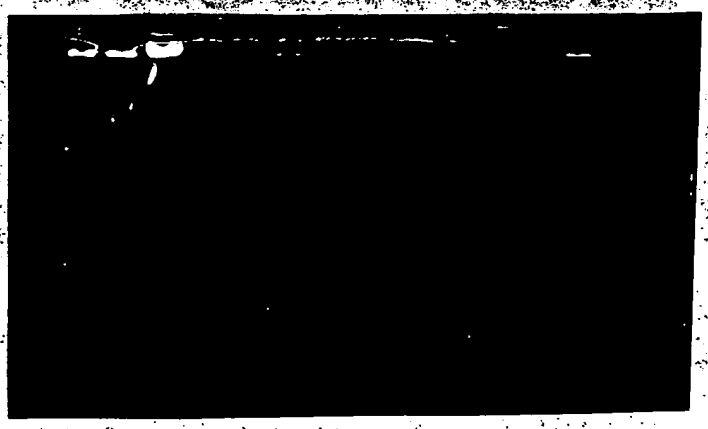
22 V O/N

Standard transfer to prehyb for 8 hrs





P2-1 probe reading ~3x10^5 used all (~100λ)



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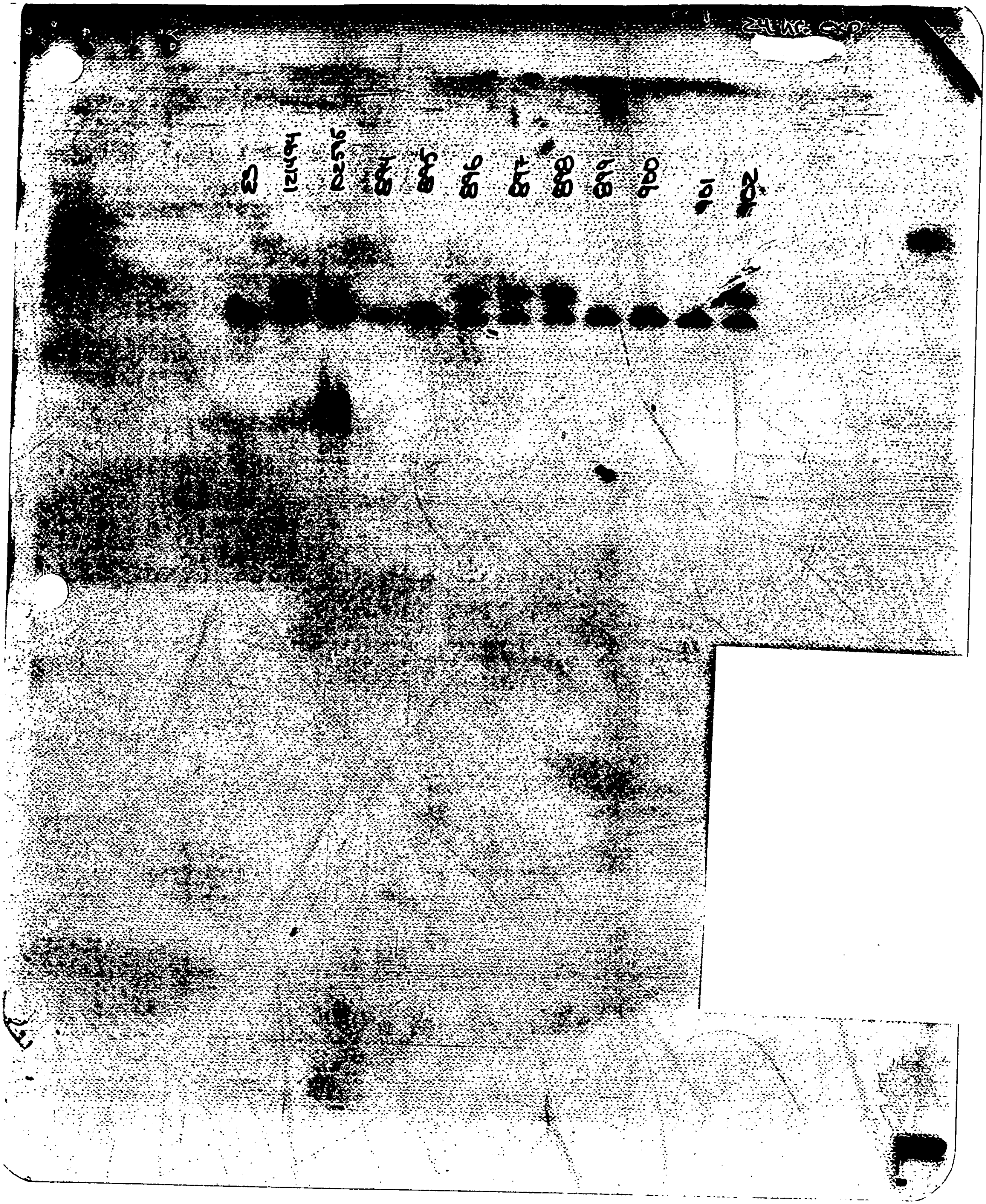
Blots of Embryos 894 to 914

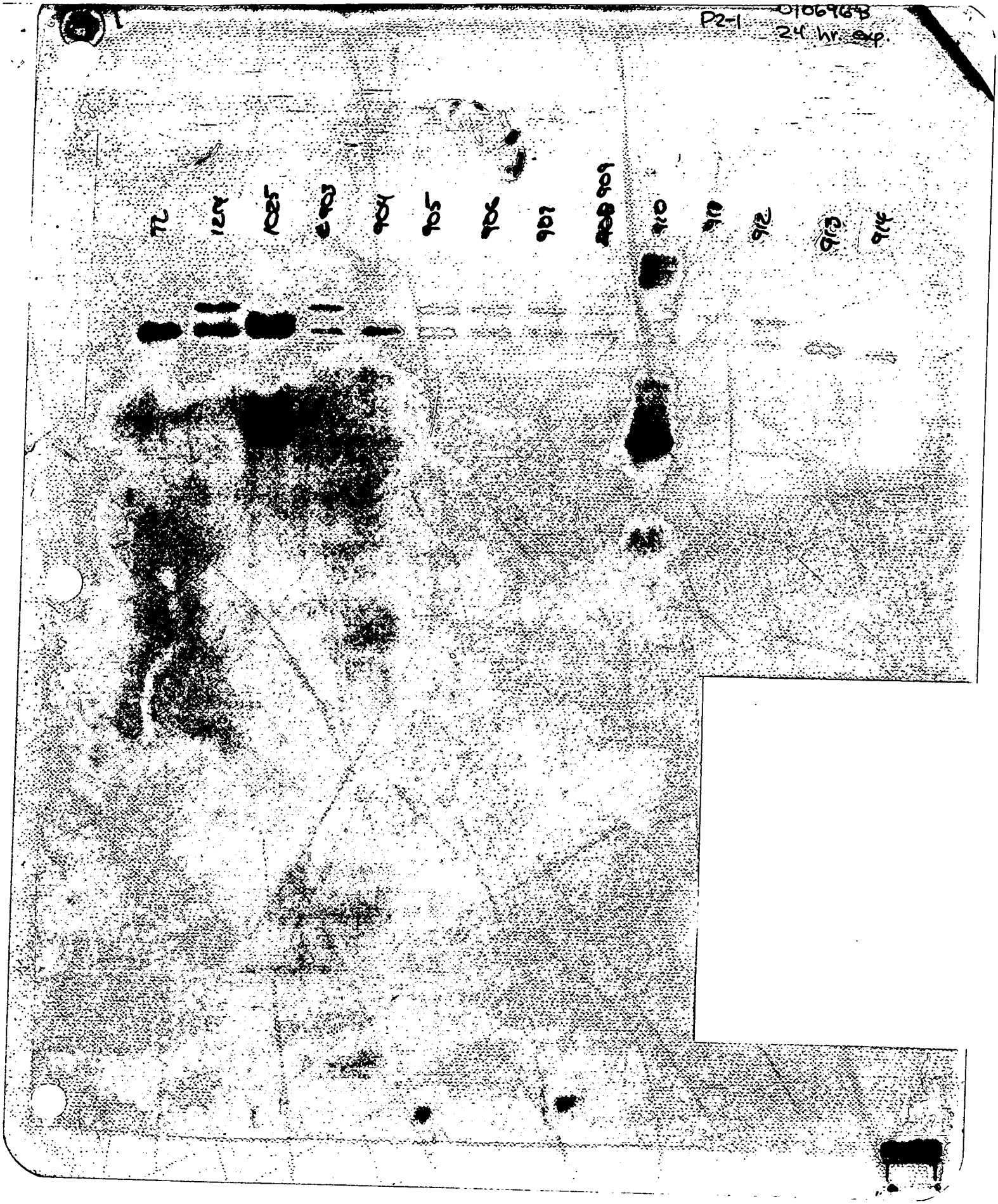
• Today Nicky developed 20 hour exposures of blots 010696 A and B after these had been probed with a Cre probe . The signal was very strong and the A blot was very clean. The910 sample of the B blot had an enormous signal in the 910 lane, which was the suspicious lane in the last probing with ß-GAL. It would appear to me that somehow the sample that was loaded into this lane was contaminated with a Cre plasmid.

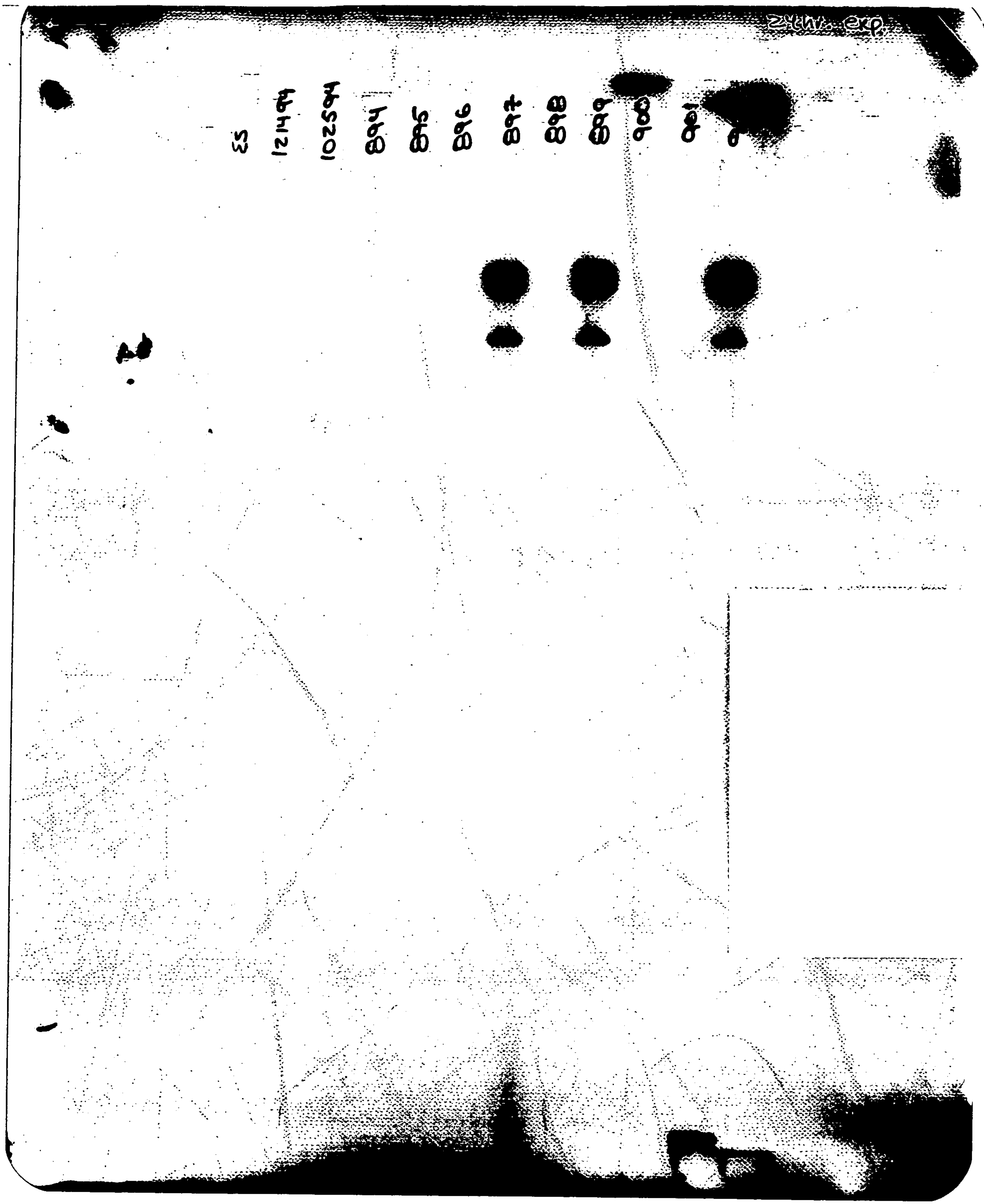
• The blot establishes that not all embryos shoing the P2Br allele also contain the procre gene.

• The blots were discarded

Embryo #	Pol II	Pro Cre	ß-GAL Stain
894	+ / +	+ / +	NEG
895	+ / +	+ / +	NEG
896	+/P2Br	+ / +	POS
897	+/P2Br	+/304	POS
898	+/P2Br	+ / +	POS
899	+ / +	+/304	NEG
900	+ / +	+ / +	NEG
901	+ / +	+ / +	NEG
902	+/P2Br	+/304	POS
903	+/P2Br	+ / +	POS
904	+ / +	+/304	NEG
905	+/P2Br	+ / +	POS
906	+/P2Br	+ / +	POS
907	+/P2Br	+ / +	POS
908	?	?	POS
909	+/P2Br	?	POS
910	+/P2Br	?	POS
911	+/P2Br	+ / +	POS
912	+/P2Br	+/304	POS
913	+ / +	+/304	NEG
914	+ / +	+/304	NEG
TOTAL			







(8)/39

24 hr. cap

Mouse Embryo Genotyping-Southern Blots

Nicky developed the films of Min's blots that had been up to hyb since yesterday. These blots (**010696 A** and **010696B**) contained samples of genomic DNA from embryos that were stained for ß-GAL activity and which have been scored previously, see above. The blots were reasonably clean and the signal was strong.

Blot **010696A** contained samples from embryos #'s **894* to 902***

- this blot contained the above samples and control DNA's from normal es cells, the original recombinant, and the Cre recombinant.
- None of the mouse samples showed the band expected for the original recombinant, they either showed only the wt allele or the wt allele and one copy of the Cre recombinant allele. The four animals showing the Cre recombinant allele were those that had been scored as ß-GAL positive previously (#896, 897, 898, and 902).

Blot **010696B** contained samples from embyos #'s **903 to 914** with the exception of **908**, a sample that was lost during DNA extraction.

- 8 of 11 samples on the blot showed the pattern of hybridization anticipated for the Cre recombined allele (903, 905, 6, 7, 9, 910, 911, 912). Three animals only showed the pattern associated with the wt allele (904, 913, 914).
- all of the animals that I had previously scored as ß-GAL positive showed the Cre recombined band on this blot.
- Sample 910 poses a real problem. In addition to the wt band and the Cre recombined band this animal shows a band of the same size as the original recombinant and some additional apparently poorly digested DNA. The wt and p2br bands are present in approximately equimolar amounts, as would be expected, and they are of approximately the same intensity as the other samples. The additional p2bc band is stronger than either of these two, and would therefore have to represent an amplification of the unrecombined allele if it were to be real. It may well be that the expression of Cre in spermatids leads to some unusual rearrangements in addition to the anticipated rearrangements. Before getting too excited about this prospect it would be useful to extract DNA from the ß-GAL-stained carcass if possible and to determine if this DNA shows the same pattern.

Friday,

Scoring embryos 903-914

903: I score this embryo as clearly positive, although the uniformity of staining is clearly disappointing. The most intense staining is in the GI tract and mouth structures, but there is clear staining of the somites as well. The vibrissal folicals are positive and the choroid plexus is also positive.

904: This embryo is entirely negative.

905: I score this animal positive with some reservations. The embryo was bisected coronally at the level of the forelimb. One side of the brain, viewed through the skin, is clearly positive. The other does not appear to be so. Some of the GI tract looks to be clearly positive, and the vibrissal follicles are faintly positive.

906: I score this embryo as positive in much the same sense as for 903. Some parts of the nervous system are reasonably well stained.

907: This embryo is very clearly positive. The cns is well-stained as are the somites. Even in this embryo there is very considerable variation in the intensity of staining from region to region.

908: This embryo is positive in the 903 sense. The cns is well, but variably, stained. The vibrissal barrels are faintly stained.

909: This embryo is clearly positive. The staining is intense in many places but it is blotchy.

910: This embryo is positive in the 903 sense.

911: This embryo is positive in the 903 sense.

912: This embryo is positive in the 903 sense, with some striking vibrissal staining and good forebrain staining.

913: This animal is clearly negative.

914: This animal is clearly negative.

894-902 Positives = 896, 897, 898, and 906

Friday,

Blots of Embryos 894 to 914

• Today Nicky developed 20 hour exposures of blots 010696 A and B after these had been probed with a Cre probe . The signal was very strong and the A blot was very clean. The 910 sample of the B blot had an enormous signal. This lane gave a puzzling result in the first probing of this blot with ß-GAL; there were signals consistant with the other lanes that indicated that there was one wild-type allele and one P2Br allele, but there was additional signal. One part of this additional signal ran at about the same level as the P2Bc allele, another part of this signal was fairly high mw DNA. In the cre probing the whole lane is a smear - the kind of signal one sees in a blot of plasmid DNA when hundreds of ng of DNA are loaded. The only way to determine what is going on in this lane will be to run another gel with the newly extracted DNA from the rest of the embro.

• The blot establishes that not all embryos shoing the P2Br allele also contain the ProCre allele.

• The blots were discarded

•• Overall, there were 21 wmbryos, 12 of the 19 that could be genotyped for RNA-PolII had the P2Br allele (none had p2bc), 7 of 18 that could be geneotyped for ProCre were positive, and 13 of 21 were positive for ß-GAL. The correlation between ß-GAL staining and the P2Br allele is 1:1.

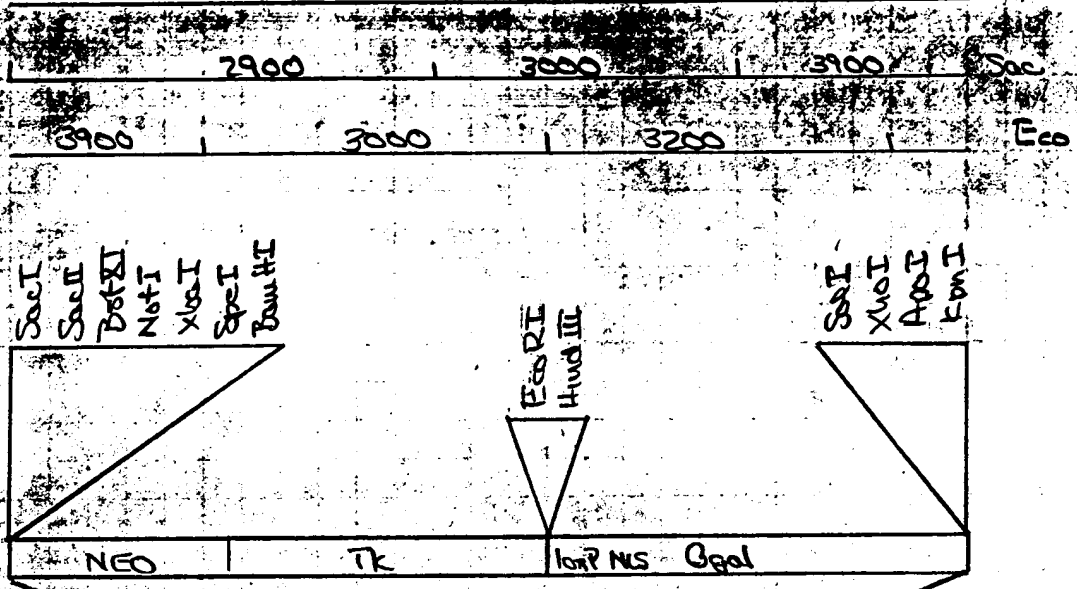
Embryo #	Pol II	Pro Cre	ß-GAL Stain
894	+/+	+/+	NEG
895	+/+	+/+	NEG
896	+/P2Br	+/+	POS
897	+/P2Br	+/304	POS
898	+/P2Br	+/+	POS
899	+/+	+/304	NEG
900	+/+	+/+	NEG
901	+/+	+/+	NEG
902	+/P2Br	+/304	POS
903	+/P2Br	+/+	POS
904	+/+	+/304	NEG
905	+/P2Br	+/+	POS
906	+/P2Br	+/+	POS
907	+/P2Br	+/+	POS
908	?	?	POS
909	+/P2Br	?	POS
910	+/P2Br	?	POS
911	+/P2Br	+/+	POS
912	+/P2Br	+/304	POS
913	+/+	+/304	NEG
914	+/+	+/304	NEG

New Blot of Embryos 894-902

• The first Southern blot (010696A) of these embryos looked pretty good - the samples are appropriate for attempting to make a publication Southern. Nicky poured a new 0.6% gel and did Pst I digests of the DNA's that were on that blot, and did an additional digest of 392 DNA. The expectation is that the somatic (tail)DNA of the 392 mouse, which sired this litter, will show the P2Bc allele, while the embryos he sired will show the P2Br allele. We did not have hoe assay results for the samples of 392 that we had previously prepared so Nicky read the OD 260 and we estimated that it would be high by about 1/3, so instead of running a nominal 10γ of DNA we ran a nominal 15γ of DNA - the latter should come close to a true 10γ of DNA because the sample is heavily contaminated with RNA.

• The gel was run overnight at 24 volts to minimize band smiling. On Wednesday it will be transferred and put up to prehyb overnight - it will be probed with P2-1 overnight on Thursday

235c Is Corrected Version



Vector = pOS227 cut with HindIII and SalI

Insert = pOS230 cut with HindIII and SalI

6/45

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Digests for 235c and 239c

235
239

① HindIII digest of 227c RL #1

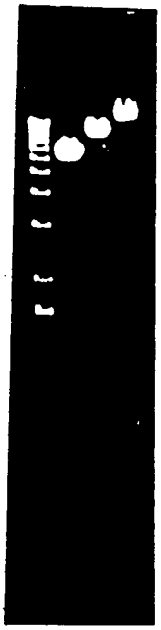
25λ DNA
20λ 10X #2
10λ HindIII
145λ H_2O

② HindIII digest pOS235

20λ DNA 10/9/93 @ 268/λ
20λ 10X #2
10λ HindIII
168λ H_2O

③ HindIII digest of 239

1.5λ DNA 12/2/93 @ 3.38/λ
20λ 10X #2
10λ HindIII
168λ H_2O



5λ each digest → minigel
balance cleaned up and ppt'd

1/11/94

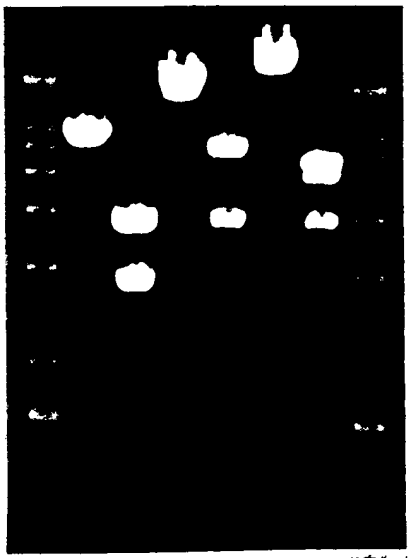
DNA from down, resuspended in 20λ, 1/1 onto minigel.

BamHI digests:

10λ DNA
5λ 10X Bam
5λ BamHI
30λ H_2O

Minigel

1/20th 227c HindIII
1/10th 227c + Bam
1/20th 235 HindIII
1/10th 235 + Bam
1/20th 239 HindIII
1/10 239 + Bam



Realized that I needed another strategy for 239c

∴ KpnI digest 239 cut with HindIII

10λ DNA
5λ 10X #1
5λ KpnI
20λ H_2O

45 minutes at 37°C, then 2λ → minigel
25λ reserved on ice
25λ for BamHI digest
+5λ 100 mM Tris
+5λ 1500 mM NaCl
2.5λ 100 mM $MgCl_2$
+2.5λ BamHI
+10λ H.O

6/16

150 × 10 = 1500
5M / 1.5 = 3.3×

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① Digest pOG230 with HindIII

have 20λ DNA from Qiagen prep of 230, from colony #4
OD 260/280 done on 1:100 dilution

SAMPLE	A320	A280	A260	280/260	260/280
1.0000	0.0070	0.3048	0.5816	0.5182	1.9296

Concentration of DNA = 2.9 g/λ. Will be isolating 3kb fragment from 6kb plasmid. Digest about 5g DNA

2λ DNA — 3λ → minigel.
20λ 10X #2 — clean and ppt balance
10λ HindIII
168λ H_2O

② Digest pOG227 with HindIII

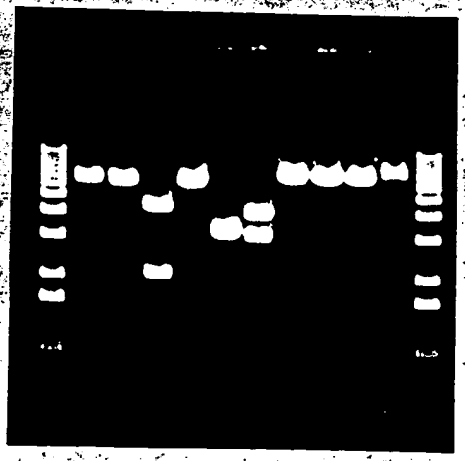
have Qiagen prep from 101 ng/93 = 168/λ

3λ DNA — 4λ → minigel
20λ 10X — clean and ppt balance
10λ HindIII
167λ H_2O

Check digests on plasmids, 100 ng each

#230 — KpnI (1)
HindIII (3)
SacI (1)
SalI (5)
EcoRI (E)

#227 — EcoRI
BamHI
HindIII
SalI
SmaI (1)



6/47

DNA's spun down, washed, resuspended in 40 λ TE

Sal I digest (2°) 230

DNA	20 λ
10x	5 λ
SalI	5 λ
H_2O	20 λ

Sal I digest (2°) 227

DNA	20 λ
10x	5 λ
SalI	5 λ
H_2O	20 λ

LMA gel: 0.6% LMA in TAE

The two frag bands of 230 was not as separated as I would have liked.

Ligations

#1 15 λ 230 gel
5 λ 227 gel

#2 15 λ blunt gel
5 λ 227 gel

5 λ of each gel ligation was used to transform 50 λ aliquots of DH5αs A&P. Standard plating. 10/15/93

8 colonies picked into 5 ml LB + Amp (plates gave very good transformation ratio. 10/17/93

Std. rapids done. Pellets up in 50 λ. 1 λ digested with EcoRI 10/18/93

Overloaded. 1, 2, 3, 4, 7 look to be correct.

#1 & 2 scaled for Qiagen preps

6/48

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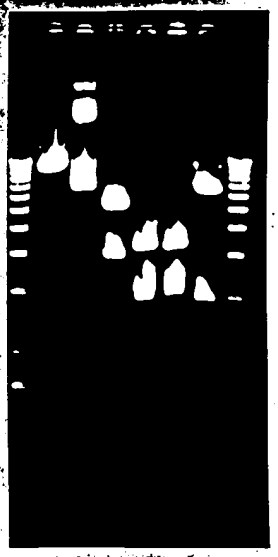

Additional checks on 235 RC #1.

1) Hind III
2) XbaI
3) Hind III + XbaI
4) SacI
5) EcoRI
6) SalI + BamHI

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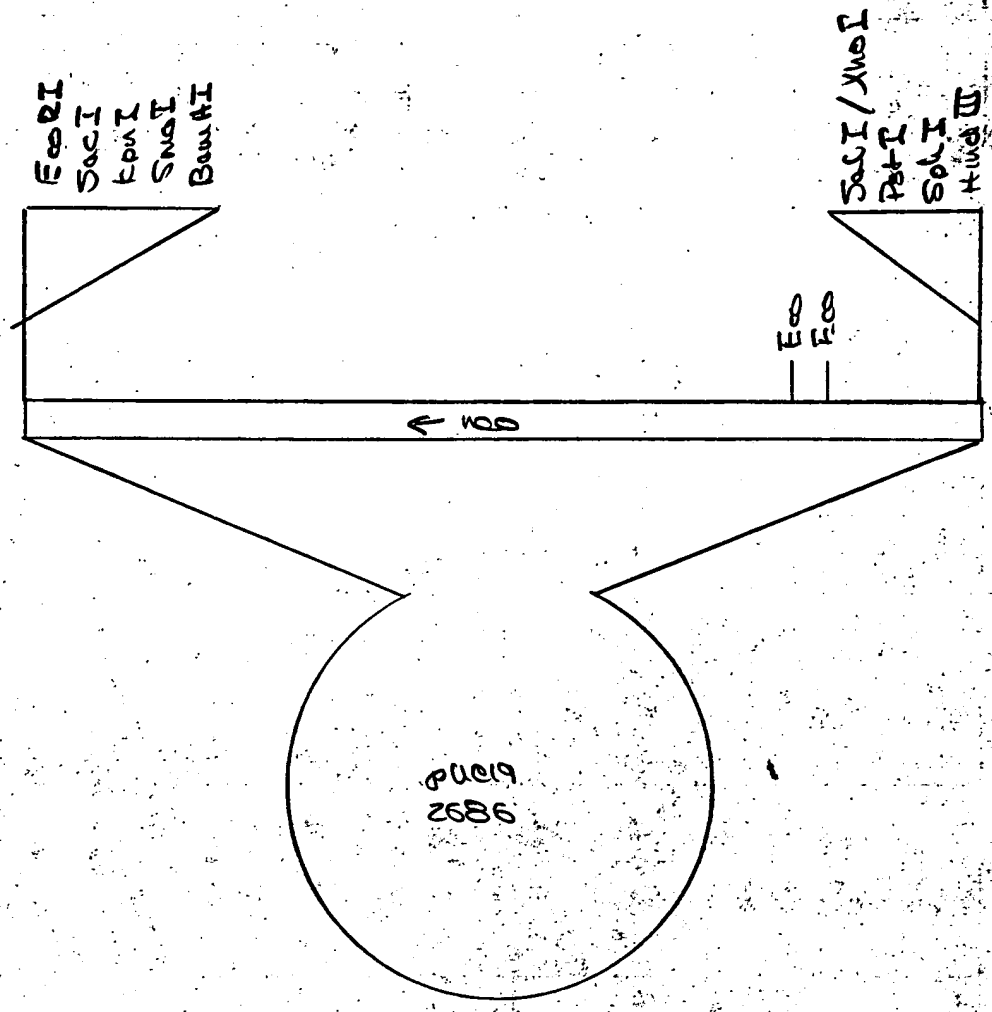
6/49

MCI was Poly A cassette into pUC19



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MCI neo PolyA → pUC19

① Digest pMCI neo with BamHI 5/28/90 prep is 5.7 g/l

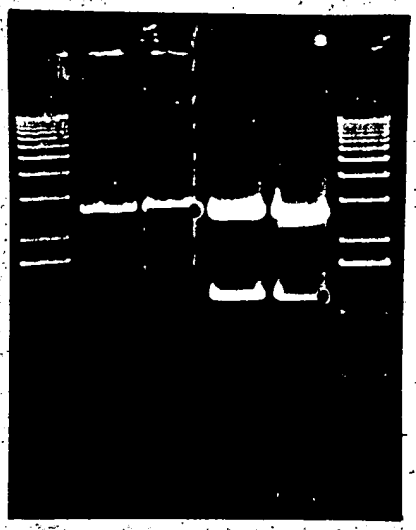
11 DNA
20 l 10x Bam
10 l BamHI
169 l H_2O

1. 229
2. pUC
3 pMC-1

② Digest pUC19 with BamHI (TP prep at 0.9 g/l)

5 l DNA
20 l 10x Bam
10 l Bam
169 l H_2O

Both digests run one hour at 37°
Extracted and ppted overnight.
Resuspended in 25 l TE
1 l → minigel.



③ Digest Bam - MCI neo with XhoI

15 l DNA
5 l 10x #2
5 l XhoI
25 l H_2O

④ Digest Bam - pUC with SacI

15 l DNA
5 l 10x Sac
5 l SacI
25 l H_2O

LMA gel: pUC19 15 l
25 l
MCI neo 15 l
35 l

Bands excised and left in reprig overnight.

12/18 Standard ligations set up

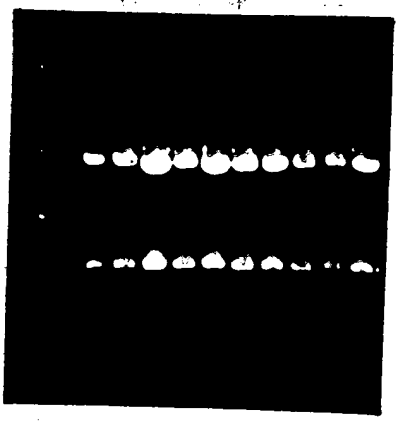
12/19 Transformed into AG-1

12/20 Good transformation robo - 10 colonies picked.

6/51

Rapids digested with EcoRI.

all colonies look to be correct #1 & #2 saved.



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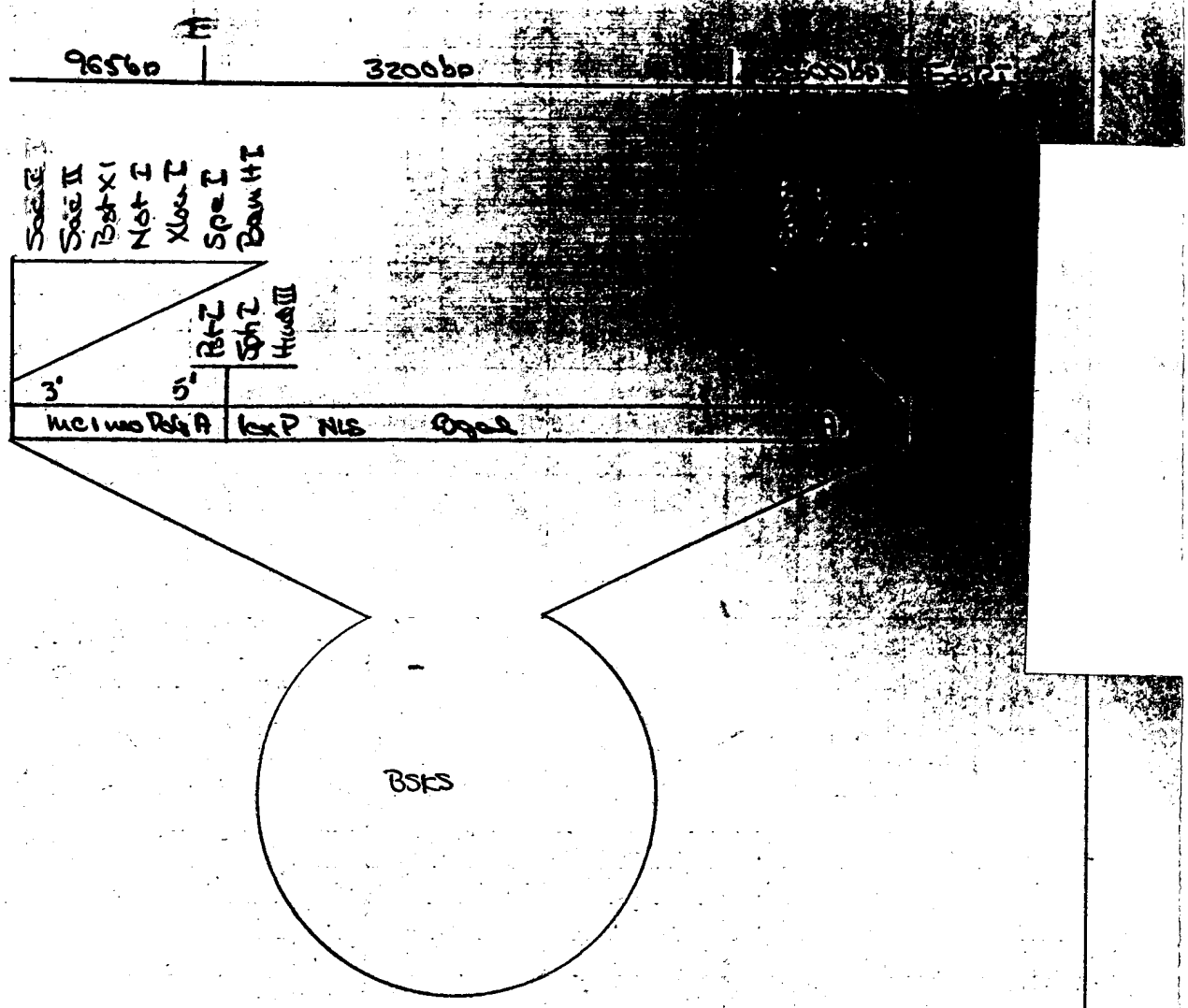
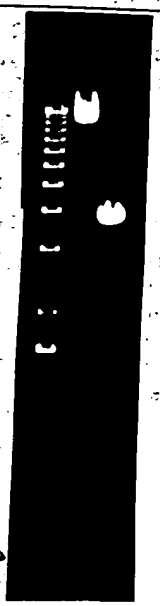


MC1neo PolyA cassette from pOG247 (known to be wild-type)
Bgal and vector sequences from pOG235

This plasmid is part of a replacement vector for the Pol II locus.

① Digest pCG247 with HindIII

2λ DNA 12/27/93 prep at 3.5δ/λ
20λ 10x#2
10λ HindIII
168λ H_2O



② Digest pCG235 with HindIII

2λ DNA (10/19/93 prep at 2.6δ/λ)
20λ 10x#2
10λ HindIII
168λ H_2O

4λ each digest loaded onto minigel → look good
DNA cleaned up and precipitated
resuspended in 20λ TE

③ BamHI of digest 247 and 235

20λ DNA
5λ Bam 10x
5λ Bam
20λ H_2O

digested one hour
2λ → minigel
20λ of 235 digest and 35λ of 247 digest loaded onto LMA gel

Bands excised and standard ligations set up.

L1		L2	
5λ	235 gel		=
15λ	247 gel	15λ	blank
12λ	10x gel ligbuff		=
1λ	100mM ATP		=
1λ	T4 ligase		=
86λ	10mM Tris		=

1/5/93 5λ aliquots of ligations were used to transform 50λ aliquots of AG-1.

1/6/93 Excellent transformation rates obtained > 10:1

b/54

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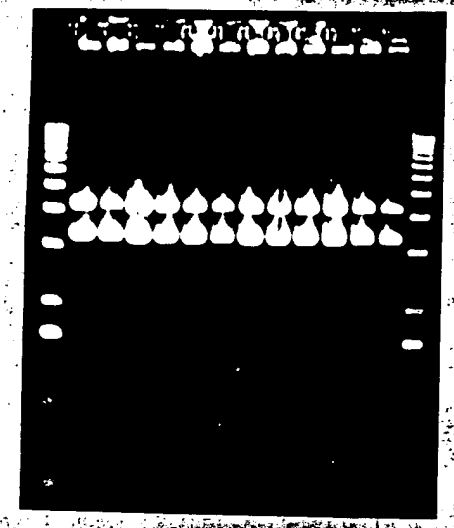
Standard boiling rapid lysates. Up in 100λ

1λ aliquots digested with EcoRI



All samples yield fragments predicted for correct recombinant.

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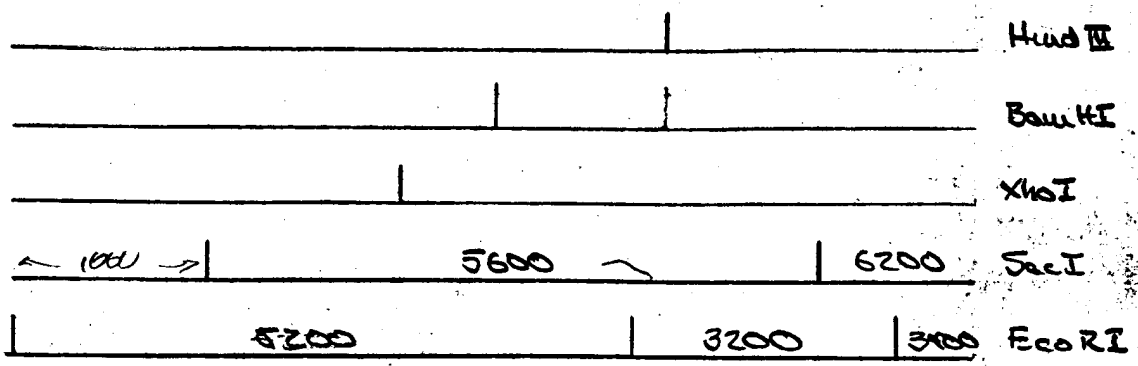
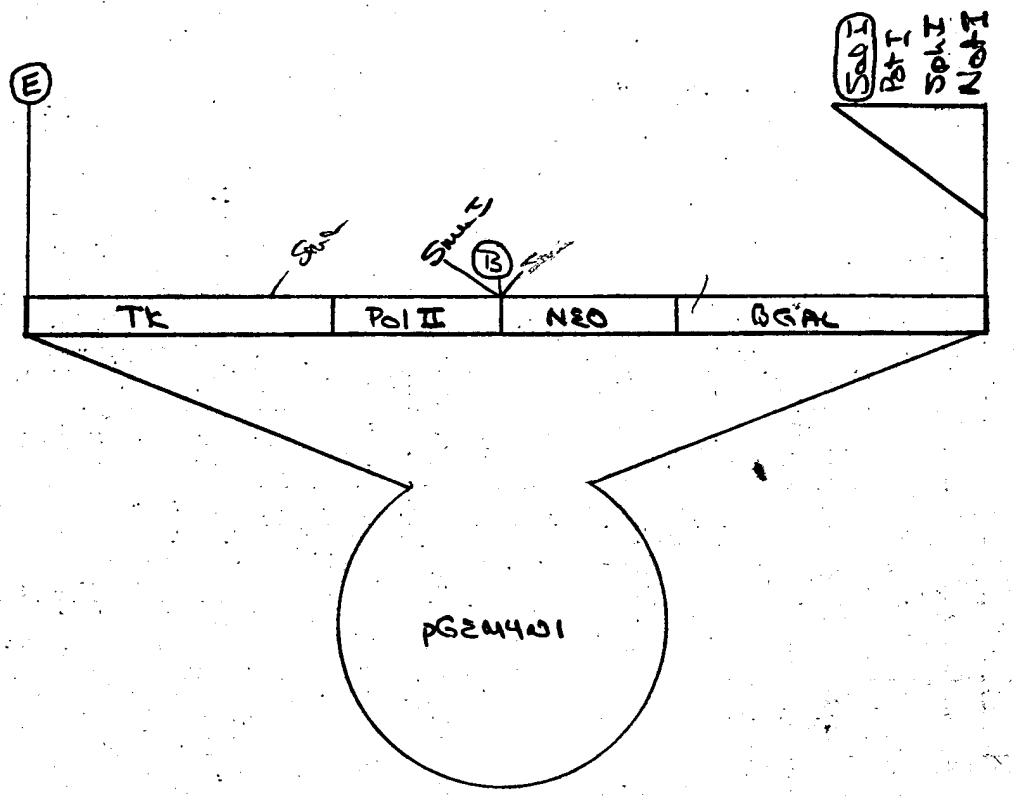
6/55

TK+PolII plus Neo + Bgal for Replacement.





EcoRI to BamHI (4000bp) from 245 T.

BamHI to SalI (4800bp) from 250

SalI + EcoRI (2900bp) from pGEM401

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① EcoRI + BamHI digest 245

10λ 245 RL #1
5λ 10X #3
2.5λ EcoRI
2.5λ BamHI
30λ H_2O

② BamHI + SalI digest 250

10λ 250 RL #1
5λ 10X Sal
3λ SalI
2λ BamHI
30λ H_2O

③ Eco + Sal digest pGEM4N1

1λ DNA
5λ 10X Sal
3λ SalI
2λ EcoRI
38λ H_2O

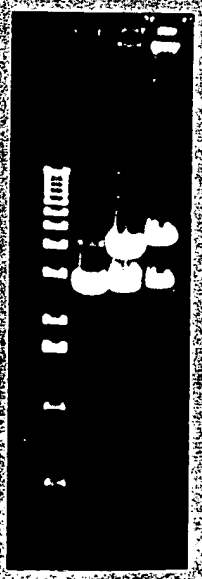
Minigel 5λ each digest loaded on minigel.

1/22/93 LMA gel 30λ of 245, 250 digests and 10 and 20λ of pGEM digest were loaded onto 0.6% LMA gel

Loaded Gel Incorrectly

Redo digests as above 5λ each digest → minigel

20λ pGEM, 30λ 245, 40λ 250 loaded on LMA gel.



Ligations:

L1 5λ pGEM
7.5λ 245
7.5λ 250

L2 5λ pGEM
15λ blank

other components standard.

1/23 10λ each ligation → 100λ AG-1

1/24 excellent ratio obtained.
12 colonies → AG-1

6/57

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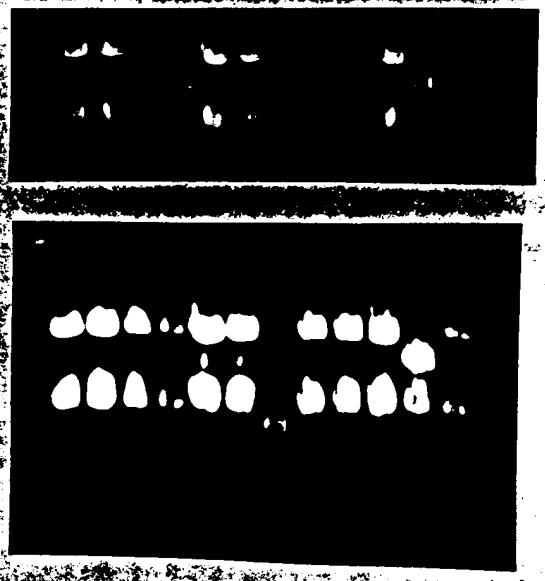
Std boiling reps - EcoRI digests:



All but 7 and 11 look correct

~3000bp doublet decreased in #2

#2 and #6 saved.

Numerous additional digests done for construction of 260 -see notes

#2 scaled for Glycerol prep 1/26

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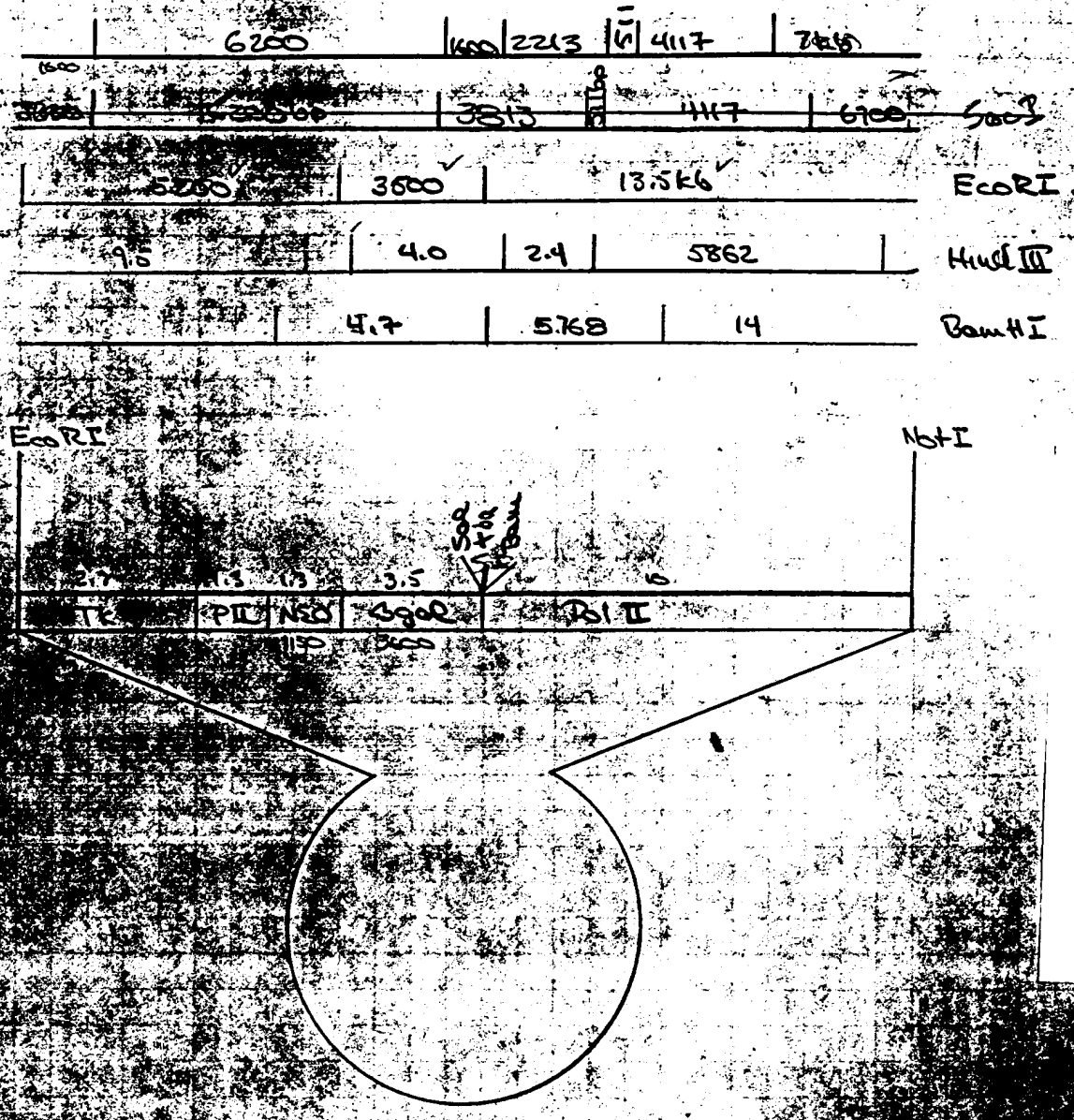
Pol II - Bgal Replacement Vector



Vector = 7254 cut with SalI and NotI. Includes TK [illegible] NOS Bgal (ca 11.4kb)

Insert = 2554 cut with SalI and NotI. Includes ca [illegible] of Pol II genomic sequence

6/59

Further checks on 254 RC #2

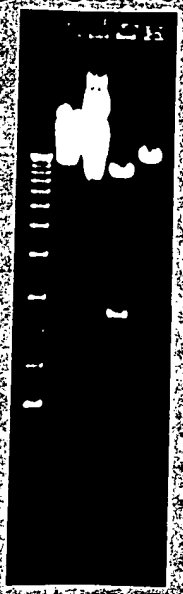
2	EcoRI	(Eco)
4	BamHI	(Bam)
1	XhoI	(#2)
3	Eco + Bam	(#3)
5	Not	(#3)
6	Sal	(Sal)
7	Sal + Bam	(Sal)

Preparative digest 254

10 λ 254 RC #2
5 λ 10x Sal
3 λ SalI
2 λ NotI
30 λ H_2O

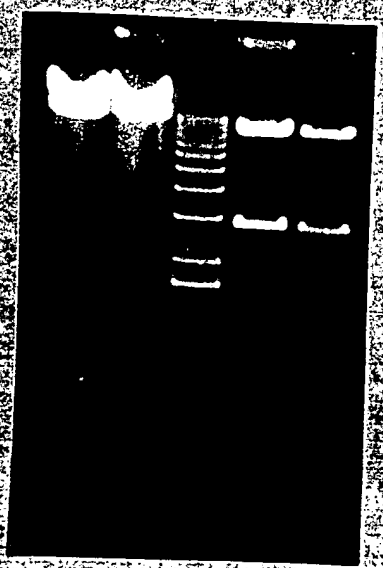
Preparative digest 255N

11 λ 255N prep 1/14/94
=
=
=
39 λ H_2O
① Add all components except Not
② Incubate 5 λ → 37°
③ Add NotI



1% LMA gel

1) 15 λ 254
2) 35 λ 254
3) 40 λ 255N
4) 10 λ 255N



1/10 254 Not + Sal
1/10 " Sal only
1/10 255N Not + Sal
1/10 255N Sal only

It appears that the SalI digest of 254 may be incomplete.

LMA gel - want to use about 2x the amount of 255N to get good concentration of fragment

New Digests.

20 λ 254 N #2 (now diluted 2x compared to previous digests)
20 λ 10x SSB
15 λ SspI
155 λ H_2O

3 λ 255 N prep 1/19/94
20 λ 10x SSB
10 λ SspI
167 λ H_2O

1, 3, 6 λ of each digest

digests cleaned up and spotted overnight.
resuspended in 20 λ TE.

1/27/94

- 2.6 and 10 λ aliquots of 1° digests were redigested with NotI in Vt = 40 λ, with 4 λ enzyme each.

- after one hour digestion each digest was loaded onto a 0.6% TAE LMA gel.
Run at 30V

Indicated bands excised (photo of post excision)

Ligations:

L1	L2
3 λ 254 gel	3 λ 254
7 λ 255N gel	7 λ 255 N
	+ 6 λ 10x
	+ 1 λ ATP
	+ 1 λ ligase
	+ 40 λ 0.5 mM Tris

1/28/94 10x each ligation was used to transform 25 λ aliquots of AG1

1/29/94 Obtained approximately 10 L1 L2 colonies

1/30/94 12 colonies picked into LB + Amp.

1/31/94 Std. mini's digested with HindIII

all look correct.
#1 #2 saved
#1 restreaked, scaled.

36 ♂ ? Southern not clear sac — p2-5 F_0 ♂ #9 × C57BL/6
10 b 6-24-95

37 ♂ +/− all Agouti

38 ♀ +/−

39 ♀ +/−

40 ♀ +/−

41 ♀ +/+ sac

42 ♂ +/+

43 ♀ +/+

44 ♀ +/+

45 ♂ +/+

C57BL/6

	46	♀	+/+		p2·5 F_0 ♂ 7x C57BL/6 ♀
	47	♂	+/+		7 b
	48	♀	+/+		tails
→	49	♂	+/-	mouse with Rib punch sized 439-453	All Agouti
	50	♀	+/+	0/13 positive	
	51	♀	+/+	SAC	
	52	♀	+/+		
✓	53	♀	+/+	SAC	304 M F_0
✓	54	♀	+/+	SAC	6 b
✓	55	♀	+/+	SAC	tails
✓	56	♂	SAC 8/15	+/+	1/6
✓	57	♂	SAC 8/15	+/+	
✓	58	♀	+/304M		

7/8/63

59	♂	+/+	SAC		P2-5 F_1
✓ 60	♀	+/−	SAC		P2-5 F_0 ♂#9 × C57BL/6 ♀
61	♂	+/+			7 b
62	♀	+/+	SAC		(9 total in litter, 2 died)
63	♀	+/+	SAC		All agouti
64	♂	+/+	SAC		
✓ 65	♀	+/−	SAC		
66	♀	+/+	A/	SAC	P2-5 F_1
67	♂	+/+	A/		P2-5 F_0 ♂#9 x C57BL/6 ♀
					2 b ; tails
68	♂				304 M F_0 (129 SV)
69	♂				3 b
70	♀		+/304M		tails
71	♀	B/- A/-	+/304M	SAC 12/12 no good as sire	304 M F_0
72	♂	-/- -/- c/c	+/304M	SAC 12/11/95	9 b
73	♂	B/- A/-	SAC		tails
74	♂	-/- -/- c/c	SAC		
75	♂	B/- A/-	+/304M	SAC	
76	♂	-/- -/- c/c	SAC		
77	♀	-/- -/- c/c	SAC		
✓ 78	♂	b/b a/9	+/304M	(high copy number)	SAC
79	♀	b/b A/-	SAC		

71 8/64

80	♀ B/- A/-	Sac	304 M F_0
81	♂ B/- A/-	↓	10 b
82	♀ -/- -/- c/c	↓	tails
83	♂ B/- a/a	+/304M ✓ Sac	
84	♂ B/- A/-	Sac	
85	♂ -/- -/- c/c	↓	
86	♂ B/- A/-	? +/304M (signif low)	Sac 8/22
87	♂ b/b a/a	Sac	
88	♀ B/- A/-	↓	
89	♀ B/- A/-	↓	
	8-7-95		
90	♀ B/- A/-		304 M F_0
91	♂ -/- -/- c/c		15 b
92	♂ B/- A/-		
93	♀ -/- -/- c/c		
94	♂ B/- A/-		
95	♂ b/b A/-		
96	♀ B/- A/-	+/304M ✓ Sac	(high copy number)
97	♂ B/- A/-		
98	♂ -/- -/- c/c		
99	♀ B/- a/a	+/304M ✓	
100	♂ B/- A/-		
01	♂ B/- a/a		
02	♂ b/b A/-		7/8/95
03	♀ B/- A/-	+/304m ✓	(high copy #)
04	♂ b/b a/a		

884 +/+

5 +/+

P2-5 304

886 +/+ +/+ 304MK P2-5

7 +/P2-5 ♀ 547 (+/304M, B)

8 +/P2-5 ♂ 503 (+/P2-5)

9 +/P2-5 plug

890 +/P2-5 harvest

1 +/+ blot 123095A

2 +/+ +/304

3 +/+ +/304

4 +/+ +/+

895 a/a PBC-118

6 F_0♀243 × F_0♂241

7 SOC 96

8

900

1

2

3 d. ?

4

	P2.5		
894*	+/+	-	304 - P2B
5*	+/+	-	CBGF, ♀
6*	+/P2Br	-	plugged by
7*	+/P2Br	+/304	304M/P2B o? 392
8*	+/P2Br	-	harvested
9*	+/P2Br	+/304	Blot 010686A
900*	+/+	-	
1*	+/+	-	
2	+/P2Br	+/304	
903	+/P2Br		304 - P2B
4	+/+	+/304	CBGF, ♀
5	+/P2Br	-	plugged
6	+/P2Br	-	by 304M/P2B o? 519
7	+/P2Br	-	harvested
8	+/P2Br	-	blot 010695B
9	+/P2Br	-	blot 011796
910	+/P2Br ← ???	-	
1	+/P2Br	-	
2	+/P2Br	+/304	
3	+/+	+/304	
4	+/+	+/304	

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